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(54) Title: SYSTEM FOR REPRODUCING AND MODULATING STABILITY AND TURNOVER OF RNA MOLECULES

(57) Abstract

An *in vitro* system is provided that recapitulates regulated mRNA stability and turnover of exogenous RNA substrates. The system comprises a cell extract optionally depleted of activity of proteins that bind polyadenylate, and a target RNA sequence. This system is used for the identification of agents capable of modulating RNA turnover, as well as agents capable of modulating RNA turnover in the presence of RNA stability modifying agents.

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SYSTEM FOR REPRODUCING AND MODULATING STABILITY AND TURNOVER OF RNA MOLECULES

GOVERNMENTAL SUPPORT

The research leading to the present invention was supported, at least in part, by grant No. GM56434 from the National Institutes for Health. Accordingly, the Government may have certain rights in the invention.

FIELD OF THE INVENTION

Broadly, the present invention involves a system and method for monitoring the stability of RNA and identifying agents capable of modulating RNA stability.

BACKGROUND OF THE INVENTION

The relative stability of a mRNA is an important regulator of gene expression. The half-life of a mRNA plays a role in determining both the steady state level of expression as well as the rate of inducibility of a gene product. In general, many short-lived proteins are encoded by short-lived mRNAs. Several mRNAs that encode stable proteins, such as α-globin, have also been shown to have extraordinarily long half-lives. Surveillance mechanisms are also used by the cell to identify and shorten the half-lives of mRNAs that contain nonsense codon mutations. Clearly, changes in the half-life of a mRNA can have dramatic consequences on cellular responses and function.

Little is known about mechanisms of mRNA turnover and stability in mammalian cells, but *in vivo* data are beginning to allow some generalizations about major pathways of mRNA turnover. The mRNA poly(A) tail can be progressively shortened throughout the lifetime of a mRNA in the cytoplasm. Controlling the rate of this deadenylation process appears to be a

target for many factors that regulate mRNA stability. Once the poly(A) tail is shortened to approximately 50-100 bases, the body of the mRNA is degraded in a rapid fashion with no discernible intermediates. The process of translation also influences mRNA stability. Little is known, however, concerning the enzymes and regulatory components involved in mammalian mRNA turnover.

Several cis-acting elements have been shown to play a role in mRNA stability. Terminal (5') cap and 3'-poly(A) structures and associated proteins are likely to protect the transcript from exonucleases. Several destabilizing as well as stabilizing elements located in the body of the mRNA have also been identified. The best characterized instability element is an A-U rich sequence (ARE) found in the 3' untranslated region of many short-lived mRNAs. These AREs primarily consist of AUUUA (SEQ ID NO: 12) repeats or a related nonameric sequence. AREs have been shown to increase the rate of deadenylation and mRNA turnover in a translation-independent fashion. For example, proteins with AU-rich elements include many growth factor and cytokine mRNAs, such as c-fos, c-jun, c-myc TNFα, GMCSF, IL1-15, and IFN-β. Other stability elements include C-rich stabilizing elements, such as are found in the mRNAs of globin, collagen, lipoxygenase, and tyrosine hydroxylase. Still other mRNAs have as yet uncharacterized or poorly characterized sequence elements, for example, that have been identified by deletion analysis, e.g. VEGF mRNA.

Numerous proteins have been described that interact with some specificity with an ARE, but their exact role in the process of mRNA turnover remains to be defined. For example, proteins which bind to the ARE described above include HuR and other ELAv family proteins, such as HuR (also called HuA), Hel-N1 (also called HuB), HuC and HuD; AUF1 (four isoforms); tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B. Many others have not been extensively characterized.

Through the application of genetics, the mechanisms and factors involved in the turnover of mRNA in *Saccharomyces cerevisiae* are beginning to be identified. One

major pathway of mRNA decay involves decapping followed by the action of a 5'-to-3' exonuclease. Evidence has also been obtained for a role for 3'-to-5' exonucleases in an alternative pathway. Functionally significant interactions between the cap structure and the 3' poly(A) tail of yeast mRNAs have also been described. Several factors involved in the translation-dependent pathway of nonsense-codon-mediated decay have also been identified. Whether these observations are generally applicable to mammalian cells, however, remains to be established.

Mechanistic questions in mammalian cells are usually best approached using biochemical systems due to the inherent difficulties with mammalian cells as a genetic system. Thus, efforts have been made to develop *in vitro* systems to study mRNA stability and turnover. However, the presently available *in vitro* systems suffer from numerous limitations. For example, many suffer from poor data quality and a general lack of reproducibility that significantly limits their application. Another key problem is that most of these systems do not faithfully reproduce all aspects of mRNA stability. A significant difficulty in the development of these systems is to differentiate between random, non-specific RNA degradation and true, regulated mRNA turnover. The significance of all previous *in vitro* systems to the true *in vivo* process of mRNA stability, therefore, is unclear. To date, no *in vitro* mRNA stability system has been generally accepted in the field as valid and useful. Other problems that have been uncovered in presently available systems are that they usually involve a complicated extract protocol that is not generally reproducible by other laboratories in the field. Also, presently available systems can only be used to assess the stability of endogenous mRNAs, severely limiting their utility. Finally, the data quality obtained using such systems is highly variable, precluding their use in sensitive screening assays.

Accordingly, there exists a need for an *in vitro* RNA stability system is efficient and highly reproducible, and further, one which produces minimal to undetectable amounts of RNA degradation

A further need exists for an *in vitro* RNA stability system wherein deadenylation of an RNA transcript in the system should occur before general degradation of the mRNA body is observed. Also needed is an *in vitro* RNA stability system wherein degradation of the mRNA body occurs in an apparently highly processive fashion without detectable intermediates, and further, the regulation of the rate of overall deadenylation and degradation should be observed in a sequence-specific manner. Such a system should be applicable to exogenous RNAs and allow ease of experimental manipulation.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

SUMMARY OF THE INVENTION

In accordance with the present invention, an *in vitro* system for modulating the stability and turnover of an RNA molecule is provided which models RNA processing in vivo. Thus, the present invention permits high throughput screening of compounds/macromolecules that modulate the stability of eukaryotic RNAs in order to identify and design drugs to affect the expression of selected transcripts, as well as to aid in the characterization of endogenous proteins and other macromolecules involved in mRNA stability. The in vitro system of the present invention is useful as a diagnostic aid for determining the molecular defect in selective disease alleles; development of *in vitro* mRNA stability systems for other eukaryotic organisms including parasites and fungi which should lead to novel drug discovery; and improving gene delivery systems by using the system to identify factors and RNA sequences that affect RNA stability.

Broadly, the present invention extends to an *in vitro* system capable of recapitulating regulated RNA turnover of an exogenously added preselected target RNA sequence, the system comprising a cell extract and a target RNA sequence. In a non-limiting example of

the system described herein. the regulated RNA turnover is AU-rich element regulated RNA turnover or C-rich element regulated RNA turnover.

The cell extract of the system of the present invention is isolated from lysed eukaryotic cells or tissues: the cell extract may be obtained for example from a cell line. such as HeLa cells or a T cell line. but the invention is not so limited. The cell extract may be prepared from cells comprising foreign nucleic acid. such as those that are infected. stably transfected, or transiently transfected. The cell extract may be partially purified.

In one embodiment of the invention. the cell extract may be depleted of activity of proteins that bind polyadenylate. The depletion of activity of proteins that bind polyadenylate from the cell extract may be achieved by any of a number of methods. for example, the addition to the system of polyadenylate competitor RNA: the sequestration of proteins that bind polyadenylate; the addition of a proteinase that inactivates a protein that bind to polyadenylate: or addition of an agent that prevents the interaction between polyadenylate and an endogenous macromolecule that binds to polyadenylate. to name a few. As further examples of the methods for sequestration of proteins that bind polyadenylate. it may be achieved by such non-limiting procedures as the treatment of the extract with an material that depletes macromolecules that bind polyadenylate. such as antibodies to proteins that bind polyadenylate. polyadenylate. and the combination. The material may be attached to a matrix. Other methods to achieve the depletion of the activity of proteins that bind polyadenylate may be used.

The target RNA sequence used in the system may be, by way of non-limiting examples. synthetic RNA. naturally occurring RNA. messenger RNA. chemically modified RNA, or

RNA-DNA derivatives. The target RNA sequence may have a 5' cap and a 3' polyadenylate sequence. The target RNA sequence may be unlabeled target RNA sequence, labeled target RNA sequence, or a the combination of both. The labeled RNA sequence may be labeled with a moiety such as, but not limited to a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties. Other moieties and means for labeling RNA are embraced herein.

The system of the present invention may additionally include exogenously added nucleotide triphosphate; ATP is preferred. It may also include a reaction enhancer to enhance the interaction between the various components present in the system, for example, polymers such as but not limited to polyvinyl alcohol, polyvinylpyrrolidone and dextran; polyvinyl alcohol is preferred.

The present invention is also directed to a method for identifying agents capable of modulating the stability of a target RNA sequence. The method is carried out by preparing the system described above which includes the cell extract depleted of activity of proteins that bind polyadenylate and the target RNA sequence; introducing into the aforesaid system an agent to be tested; determining the extent of turnover of the target RNA sequence by, for example, determining the extent of degradation of the labeled target RNA; and then identifying an agent which is able to modulate the extent of RNA turnover as capable of modulating the stability of the target RNA sequence.

The method described above may additionally include nucleotide triphosphate, ATP being preferred. The agent to be tested may be, but is not limited to, an RNA stability modifying

molecule. The non-limiting selection of the types of target RNA sequence and the non-limiting types of labels useful for the RNA as described hereinabove.

The method of the present invention is useful for identifying agents which can either increases or decrease the stability of said target RNA sequence. Such agents may be capable of modulating the activity of an RNA binding molecule such as, but not limited to, C-rich element binding proteins and AU rich element binding proteins, examples of the latter including HuR and other ELAv family proteins, such as HuR , Hel-N1, HuC and HuD; AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B. This list is provided as illustrative of the types of molecules that may be evaluated in the present invention, but is by no means limiting.

In a further embodiment of the present invention, a method is provided for identifying an agent that is capable of modulating the stability of a target RNA sequence in the presence of an exogenously added RNA stability modifier or RNA binding macromolecule. Non-limiting examples of such molecules are described above. The method is carried out by preparing the system described above which includes the cell extract can be depleted of activity of proteins that bind polyadenylate and the target RNA sequence; introducing into the aforesaid system the exogenously added RNA stability modifier or binding macromolecule and the agent to be tested; determining the extent of turnover of the target RNA sequence by, for example, determining the extent of degradation of the labeled target RNA; and then identifying an agent able to modulating the extent of the RNA turnover as capable of modulating the stability of the target RNA sequence in the presence of the exogenously added RNA stability modifier.

The non-limiting selection of the components of this method are as described above. The aforementioned method is useful, for example, when the RNA stability modifier decreases the stability of said target RNA sequence, and the agent to be identified increases the stability of the target RNA sequence that is decreased by the RNA stability modifier. In addition, the method is useful when the RNA stability modifier increases the stability of the target RNA sequence, and the agent to be identified decreases the stability of the target RNA sequence that is increased by the RNA stability modifier. Non-limiting examples of RNA stability modifiers include C-rich element binding proteins, and AU rich element binding proteins, examples of AU rich element binding proteins, including HuR and other ELAv family proteins, such as HuR , Hel-N1, HuC and HuD; AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B. This list is provided as illustrative of the types of molecules that may be evaluated in the present invention, but is by no means limiting.

The present invention is further directed to a method for identifying an agent capable of modulating the deadenylation of a target RNA sequence comprising preparing the system described above in the absence of nucleotide triphosphate, such as ATP; introducing an agent into the system; and monitoring the deadenylation of the target RNA sequence. Furthermore, the invention is also directed towards a method for identifying an agent capable of modulating the deadenylation and degradation of a target RNA sequence comprising preparing the system described herein in the presence of ATP; introducing the agent into the system; and monitoring the deadenylation and degradation of the target RNA sequence. These embodiments may also be carried out in the presence of an RNA stability modifier or RNA binding macromolecule to determine the ability of the agent to modulate the effect of the modulator or binding molecule on RNA stability.

It is a further aspect of the present invention to provide a method for identifying an agent capable of modulating cell growth or cell differentiation in a mammal comprising determining the ability of said agent to modulate the stability of a target RNA sequence involved in the modulation of cell growth or differentiation in accordance with the methods described above. The agents capable of modulating cell growth or cell differentiation may intervene in such physiological processes as cellular transformation and immune dysregulation. but the invention is not so limiting.

It is yet a further aspect of the present invention to provide a method for identifying, characterizing and isolating an endogenous molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof comprising preparing the system described hereinabove: introducing a protein suspected of participating in the regulation of RNA turnover into said system: and monitoring the stability of the target RNA sequence. The endogenous molecule suspected of participating in the deadenylation and/or degradation of RNA or regulation may be protein or RNA.

In another embodiment of the invention. a method is provided for identifying an agent capable of modulating the degradation a target RNA sequence in the absence of deadenylation comprising providing a cell extract in the presence of a nucleotide triphosphate: introducing said agent into said cell extract: and monitoring the degradation of said target RNA sequence in said extract.

A further aspect of the present invention is directed to a kit for monitoring the stability of a preselected target RNA sequence under conditions capable of recapitulating regulated RNA turnover. The kit comprises a cell extract that optionally may be depleted of activity of

proteins that bind polyadenylate; other reagents; and directions for use. The kit may further comprise nucleotide triphosphates, a reaction enhancer, or both.

Accordingly, it is an object of the invention to provide a system for modulating the stability and turnover of an RNA molecule *in vitro*, which permits a skilled artisan to study the turnover generally, or deadenylation and degradation specifically, of an RNA transcript, and screen drugs which can modulate the stability and turnover of an RNA transcript. The turnover may be in the absence or presence of exogenously added RNA stability modulators, or permit the study of the role of endogenous molecules in RNA turnover.

It is another embodiment of the invention to provide a kit that a skilled artisan can readily use to modulate the stability and turnover of an RNA molecule *in vitro*, and investigate the aforementioned agents.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG.1 A-D: The addition of poly(A) to cytoplasmic S100 extracts activates specific deadenylase and degradation activities. **Panel A**. Poly(A) competitor RNA activates nucleolytic activities in the extract. A capped, radiolabeled 54 base RNA containing a 60 base poly(A) tail (Gem-A60) was incubated at 30° C with S100 extract in the absence (lanes marked S100) or presence (Lanes marked S100 +Poly(A)) of 500 ng of cold poly(A) RNA as described in Materials and Methods of Example I for the times indicated. RNA products

were analyzed on a 5% acrylamide gel containing 7M urea. The position of a deadenylated, 54 base transcript (Gem-A0) is indicated on the right. **Panel B**. The shortening of input transcripts is due to a 3'-to-5' exonuclease. Gem-A60 RNA, labeled exclusively at the 5' cap, was incubated in the *in vitro* mRNA stability system for the times indicated. Reaction products were analyzed on a 5% acrylamide gel containing 7M urea. The position of a deadenylated, 54 base transcript (Gem-A0) is indicated on the right. **Panel C**. An alternative approach also demonstrates that the shortening of input transcripts is due to a 3'-to-5' exonuclease. ARE-A60 RNA, radiolabeled at A residues, was incubated in the *in vitro* stability system for the times indicated. Reaction products were hybridized to a DNA oligo and cleaved into 5' and 3' fragments using RNase H. Fragments were analyzed on a 5% acrylamide gel containing 7M urea. **Panel D**. The 3'-to-5' exonuclease activity is a specific deadenylase. Gem-A60 RNA or a variant that contains 18 extra nucleotides after the poly(A) tract (Gem-A60-15) were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The position of a deadenylated, 54 base transcript (Gem-A0) is indicated on the left. 31±11.0% of the input Gem-A60 RNA was deadenylated/degraded in 30 min.

FIG. 2 A-E: The rate of transcript degradation in the *in vitro* system is regulated by AU-rich instability elements in a sequence-specific fashion. **Panel A.** AU-rich elements dramatically increase the rate of turnover in the *in vitro* system. Gem-A60 RNA or a polyadenylated transcript that contains the 34 base AU-rich element from the TNF-α mRNA, were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (Gem-A0 and ARE-A0) are indicated. The ARE-A60 RNA was deadenylated/degraded 6.6±0.4 fold faster than Gem-A60 RNA. **Panel B.** The AU-rich element from c-fos mRNA also functions as an

instability element *in vitro*. Gem-A60 RNA or a transcript that contains the 72 base AU-rich element from the c-fos mRNA (Fos-A60) were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (Gem-A0 and Fos-A0) are indicated. The Fos-A60 RNA was deadenylated/degraded 3.5±0.3 fold faster than Gem-A60 RNA. **Panel C**. The ability of AU-rich elements to mediate transcript instability in the *in vitro* system is sequence-specific. ARE-A60 RNA or a variant that contains a mutation at every fourth position (mt ARE-A60: see Materials and Methods) were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (ARE-A0 and mt ARE-A0) are indicated. Mutations in the ARE reduced the rate of deadenylation/degradation by 3.7±1.4 fold compared to the wild type ARE-A60 transcript. **Panel D.** The TNF-α AU-rich element mediates instability in a heterologous context. A polyadenylated 250 base RNA derived from the SV late transcription unit (SV-A60), or a variant that contains the 34 base AU-rich element from the TNF-α mRNA (SVARE-A60), were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (SV-A0 and SVARE-A0) are indicated. SVARE-A60 RNA was deadenylated/degraded 3.5±0.7 fold faster than SV-A60 RNA. **Panel E**. The AU-rich element derived from the GM-CSF mRNA functions *in vitro* on nearly a full length RNA substrate. A nearly full length version of the GM-CSF mRNA that contained an AU-rich element (GM-CSF(+ARE), or a version in which the AU-rich element was deleted (GM-CSF(-ARE), were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. GM-CSF(+ARE) was deadenylated/degraded 2.8+0.2 fold faster than the GM-CSF(-ARE) transcript.

FIG. 3 A-B: Deadenylation occurs in the absence of ATP and is regulated by AU-rich elements *in vitro*. **Panel A.** Degradation, but not deadenylation, requires ATP. SV-ARE-A60 RNA was incubated in the *in vitro* system in the presence ((+) ATP lanes) or absence ((-) ATP lanes) for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of the deadenylated SVARE-A) transcript is indicated. **Panel B.** AU-rich elements regulate the rate of deadenylation on RNA substrates which carry a physiologic length poly(A) tail. SV RNA or SV-ARE RNA (a variant that contains an AU-rich element) were polyadenylated with yeast poly(A) polymerase and species that contained tails of approximately 150-200 bases were gel purified. These RNAs (SV(A150-200) and SVARE(A150-200) were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (SV-A0 and SVARE-A0) are indicated. SVARE(A150-200) RNA was deadenylated 2.2+0.3% fold faster than the SV(A150-200) transcript.

Figure 4 A-B: The HuR protein of the ELAV family specifically binds to the TNF-α AU-rich element in the *in vitro* system. **Panel A.** Two proteins specifically interact with the TNF-α AU-rich element. Gem-A60 and ARE-A60 RNAs were radiolabeled at U residues and incubated in the *in vitro* stability system for 5 min. in the presence of EDTA (to block degradation and allow for accurate comparisons). Reaction mixtures were irradiated with UV light, cleaved with RNase A, and protein-RNA complexes were analyzed on a 10% acrylamide gel containing SDS. The approximate sizes of the cross linked proteins indicated on the right were deduced from molecular weight markers. **Panel B.** The 30 kDa protein is HuR. Radiolabeled ARE-A60 RNA was incubated in the *in vitro* RNA stability system and cross-linked to associated proteins as described above. Cross linked proteins were

immunoprecipitated using the indicated antisera prior to analysis on a 10% acrylamide gel containing SDS. The lane marked Input denotes total cross linked proteins prior to immunoprecipitation analysis.

Figure 5 A-C: While AU-rich element binding factors are important to promote RNA deadenylation and degradation, the binding of the HuR protein to AU-rich elements is not associated with AU-rich element-mediated transcript instability. **Panel A.** Competition analysis suggests that AU-rich element binding factors are required for deadenylation and degradation of transcripts. SVARE-A60 RNA was incubated in the *in vitro* stability system for 30 min. in the presence of the indicated amounts of a synthetic RNA competitor that contained the TNF-α AU-rich element (ARE comp.) or a non-specific sequence. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The position of deadenylated SVARE-A0 RNA is indicated. **Panel B.** Reaction mixtures were prepared as described in panel A with the addition of EDTA to inhibit RNA turnover. Protein-RNA interactions were analyzed by UV cross linking analysis and analyzed on a 10% acrylamide gel containing SDS. The positions of AU rich element-specific cross linked species is indicated on the left. **Panel C.** Reactions were prepared exactly as described for Panel B, except samples were immunoprecipitated using a-HuR specific antisera prior to gel electrophoresis.

FIG. 6 A-D: ELAV proteins specifically stabilize deadenylated intermediates in the *in vitro* system. **Panel A.** SVARE-A60 RNA was incubated in the *in vitro* system in the presence (lanes (+) Hel-N1)) or the absence (lanes (-) Hel-N1) of 1ug of recombinant Hel-N1 protein. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The position of deadenylated SVARE-A0 transcript is indicated. **Panel B.** SVARE-A60 RNA was incubated

in the *in vitro* system in the presence of 1ug of recombinant Hel-N1 (lanes (+) Hel-N1), GST only (lanes (+) GST), or an unrelated RNA binding protein hnRNP H' (lanes (+) hnRNP H'). RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The position of deadenylated SVARE-A0 transcript is indicated. **Panel C.** ARE-A60 RNA, or an unrelated transcript that lacked an AU-rich element (CX-A60), were incubated in the *in vitro* stability system for 30min. in the presence (+ lanes) or absence (- lanes) of ~1 ug of Hel-N2 protein. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts are indicated. **Panel D.** A variant of SV-A60 RNA that contained the TNF-α ARE in the 5' portion of the transcript (SV5'AGE-A60) was incubated in the in vitro system for 50 min in the absence (- lane) or presence (+ lane) of 1 μg of Hel-N2 protein. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of imput and deadenylated transcripts are indicated.

DETAILED DESCRIPTION OF THE INVENTION

Numerous terms and phrases are used throughout the instant Specification. The meanings of these terms and phrases are set forth below.

In particular, as used herein "half-life" of an RNA molecule refers to the measurement of the decline in the amount of an RNA molecule to serve as a template for the synthesis of its protein product.

As used herein "turnover" refers to the degradation of an RNA molecule. Turnover comprises deadenylation and degradation.

As used herein a "cap" or"5' cap" or "terminal cap", and be used interchangeable, and refer to a 7-methyl guanosine (7mG) cap chemically conjugated to the most 5' nucleotide of the RNA molecule.

As used herein, the phrase "polyadenylic acid (poly(A)) tail" refers to a string of contiguous adenylic acids (polyadenylate) added post transcriptionally to the 3' end of an RNA molecule, such as mRNA.

As used herein, the term "stability" refers to the maintenance of an RNA molecule so that it can function, and thus retard the degradation process of an RNA molecule.

As used herein, the phrase "a polyadenylic acid competitor nucleic acid oligomer" refers to an oligomer comprising contiguous adenylic acids" which can be added to a system of the invention and sequester proteins that bind poly(A). Thus, the degradation of a particular RNA molecule having a poly(A) tail can be modulated.

Also, as used herein, the phrase "restriction endonuclease" refers to an enzyme that recognizes specific nucleotide sequences in a nucleic acid molecule, and produces a double-stranded break within or near the site. Some restriction enzymes, such as *Eco*RI or *Hind*III produce "complementary tails" on each of fragments produced. These tails are said to be "sticky" because under hybridization conditions they can reanneal with each other. Thus, if two separate nucleic acid molecules share the same restriction site, then both will contain complementary single-stranded tails when treated with the same restriction endonuclease, and can be spliced together forming a recombinant nucleic acid molecule.

Naturally, as used herein, the phrase "restriction endonuclease site" refers to a specific nucleotide sequence that is recognized by a specific restriction endonuclease.

Furthermore, numerous conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art can be readily utilized to practice the instant invention. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control.

A "cassette" refers to a segment of a nucleic acid molelcule, such as DNA or RNA, that can be inserted into a vector at specific restriction sites. The segment of the nucleic acid

molelcule may encode a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. Preferably, the transforming DNA should be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine: "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine: "DNA molecules"), or any phosphoester anologs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

The present invention is based upon Applicant's discovery of a heretofore unknown system for activating regulated turnover of RNA molecules *in vitro* that surprisingly and unexpectedly permits a skilled artisan to study and to modulate the stability and thus the turnover of a RNA molecule *in vitro*. Thus, the new and useful system of the invention permits accurate and faithful reproduction of both general and regulated aspects

deadenylation and degradation of an RNA molecule, also referred to herein as recapitulating regulated RNA turnover, particularly a eukaryotic mRNA transcript. In particular, the new and useful system of the invention permits minimal amounts, preferably undetectable, of mRNA turnover, and further, deadenylation of an RNA molecule occurs in the system prior to degradation of the RNA molecule, which mimics the turnover process of RNA found *in vivo*.

The key to the development of the system and methods utilizing the system are based on the discovery that polyadenylate competitor RNA is capable of sequestering proteins that bind polyadenylate and consequently activating the deadenylase enzyme, inducing RNA turnover. As it was heretofore considered that such proteins that bind polyadenylate may contribute to RNA deadenylation, the present finding that such proteins are, in contrast, stabilizers of RNA, led to the realization that the such proteins are interacting with and inactivating destabilizing mediators in vivo. Thus, the present invention is directed to an *in vitro* system capable of recapitulating regulated RNA turnover of an exogenously added preselected target RNA sequence comprising a cell extract depleted of activity of proteins that bind polyadenylate, and a preselected target RNA sequence. In one particular embodiment, the regulated RNA turnover is that modulated by AU-rich element (ARE) regulated RNA turnover. Examples of mRNAs with AU-rich elements include those of, by way of non-limiting example, c-fos; c-jun; c-myc TNF-α, GMCSF, IL1-15, and IFN-β. As noted above, AU-rich elements are sites for binding of numerous proteins, including the ELAV family of ARE-binding proteins, such as HuR , Hel-N1, HuC and HuD; others include AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B.. In another embodiment, the regulated RNA turnover is that modulated by C-rich element (CRE) regulated RNA turnover, such elements as found in the mRNA of globin

mRNAs, collagen, lipoxygenase, and tyrosine hydroxylase. Another mRNA with an as yet uncharacterized sequence element is that of VEGF. The invention, however, is not so limiting as to the particular elements or binding proteins to these elements involved in the regulation of RNA turnover.

The cell extract of the present invention is prepared from lysed eukaryotic cells or tissues. Various methods known to the skilled artisan may be used to prepare the cell extract. Various sources of cells may be used, including fresh cells and tissues, and cells lines. Such cells may comprise foreign nucleic acid, such as in cells that are infected; or are transiently or stably transfected with a mammalian expression vector, the latter as described in more detail below. For certain purposes, for example to investigate the role of infection, and in particular intracellular infection, on RNA turnover, infected cells may be utilized as the source of the cell extract herein. Cells infected with viruses or other intracellular microorganisms such as *Listeria monocytogenes*, HTLV, herpes simplex virus, and HIV, may be employed for these particular circumstances. Furthermore, prior to preparation of the cell extract, cells may be exposed to certain chemical or other extracellular stimuli, for example, hormones, growth factors, and kinase and phosphatase inhibitors, which may alter RNA turnover, for which subsequent studies as described herein may be used to identify the induction of certain proteins involved in modulating RNA turnover, or for the identification of agents which may counteract adverse RNA turnover modulation induced by such stimuli. As will be noted in more detail below, the methods herein may be used to identify agents which may protect cells by interfering with adverse RNA turnover induced by various sources. The cell extract is preferably free of nuclei and nuclear contents and comprises cytoplasm, but this is not essential unless particular components, such as enzymes or other factors, from nuclei, interfere with the operation of the system. In a typical preparation,

which may be modified without departing from the scope of the invention, cells are grown, harvested, lysed, centrifuged for 100,000 x g for 1 hour, and dialyzed. Glycerol may be added to protect the extract if stored frozen.

As described above, a cell used to prepare the cell extract may comprise foreign DNA. An isolated nucleic acid molecule to placed in a system of the invention can initially be inserted into a cloning vector to produce numerous copies of the molecule. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives, *e.g.*, pGEX vectors, pmal-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the nucleic acid molecule into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the nucleic acid molecule are not present in the cloning vector, the ends of the molecule may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the termini of the nucleic acid molecule; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the nucleic acid molecule are generated. Preferably, the cloned nucleic acid molecule is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both *E.*

coli and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences from the yeast 2μ plasmid.

Naturally, any of the methods previously described for the insertion of an isolated nucleic acid molecule into a cloning vector may be used to construct expression vectors containing a nucleic acid molecule consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Mammalian expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, *e.g.*, any expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, such as pED (*Pst*I, *Sal*I, *Sba*I, *Sma*I, and *Eco*RI cloning site, with the vector expressing both the cloned gene and *DHFR*; *see* Kaufman, *Current Protocols in Molecular Biology*, 16.12 (1991). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (*Hind*III, *Xba*I, *Sma*I, *Sba*I, *Eco*RI, and *Bcl*I cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, such as pREP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive RSV-LTR promoter, hygromycin selectable marker; Invitrogen), pCEP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive hCMV immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (*Kpn*I, *Pvu*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, *Bam*H1 cloning site, inducible metallothionein IIa gene promoter, hygromycin selectable marker: Invitrogen), pREP8 (*Bam*H1, *Xho*I, *Not*I, *Hind*III, *Nhe*I, and *Kpn*I cloning site, RSV-LTR promoter, histidinol

selectable marker: Invitrogen), pREP9 (*Kpn*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, and BamHI cloning site, RSV-LTR promoter, G418 selectable marker: Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase: Invitrogen). Selectable mammalian expression vectors for use in the invention include pRc/CMV (*Hind*III, *Bst*XI, *Not*I, *Sba*I, and *Apa*I cloning site, G418 selection: Invitrogen), pRc/RSV (*Hind*III, *Spe*I, *Bst*XI, *Not*I, *Xba*I cloning site, G418 selection: Invitrogen), and others. Vaccinia virus mammalian expression vectors (*see*, Kaufman, 1991, *supra*) for use according to the invention include but are not limited to pSC11 (*Sma*I cloning site, TK- and β-gal selection), pMJ601 (*Sal*I, *Sma*I, *Afl*I, *Nar*I, *Bsp*MII, *Bam*HI, *Apa*I, *Nhe*I, *Sac*II, *Kpn*I, and *Hind*III cloning site: TK- and β-gal selection), and pTKgptF1S (*Eco*RI, *Pst*I, *Sal*I, *Acc*I, *Hind*II, *Sba*I, *Bam*HI, and Hpa cloning site, TK or XPRT selection).

Once a particular nucleic acid molecule, such as RNA, is inserted into a vector, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few. In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired.

Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu et al., 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

Cells useful for the preparation described herein include immortalized or partially immortalized cells which can be grown in large amounts under defined conditions, such as HeLa cells and various T-cell cell lines. Other sources include tissues, blood cells, or myeloid cells. Other sources are well within the realm of the present invention.

The cell extract of the system described herein is depleted of activity of proteins that bind polyadenylate. This may be achieved by any one or a combination of methods such as the following. While not being bound by theory, each of these methods either removes the proteins that bind polyadenylate, or inactivate the binding activity. These procedures may be applied to the cell extract as it is used in the methods described herein, or the cell extract may be treated beforehand. For example, a polyadenylate competitor RNA may be added to the cell extract to provide an irrelevant RNA sequence to which the binding proteins may bind, thus clearing the target RNA sequence of such binding proteins. In another embodiment, sequestration of proteins that bind polyadenylate may be performed. Sequestration may be achieved by adding to the cell extract or exposing the cell extract to a material that binds the aforementioned proteins, such as antibodies to proteins that bind polyadenylate, or polyadenylate sequences themselves or macromolecules comprising polyadenylate sequences which serve as binding targets for such proteins. Alternatively or in addition, these protein

binding materials may be bound to a matrix, such as agarose beads, and the cell extract passed through a column of such beads to remove the proteins which bind polyadenylate. The preparation of such beads covalently modified to comprise antibodies or RNA sequences, whether polyadenylate or sequences comprising polyadenylate, are known to the skilled artisan. Another means for reducing or eliminating such activity from the cell extract is by exposure to one or more proteinase known to inactivate a protein that bind to polyadenylate. These proteinases may be added to the extract, or bound to a matrix and exposed to the extract, after which inactivation the beads may be removed. A further means encompasses addition to the extract of an agent that prevents the interaction between polyadenylate and an endogenous macromolecule that binds to polyadenylate. These and other methods embraced by the present invention achieve the desired goal of depleting macromolecules that bind polyadenylate from the cell extract, thus allowing the cell extract in combination with the target RNA sequence to undergo in vivo-like RNA turnover. One or a combination of the aforesaid methods may be employed to reduce the level of such protein to an acceptable limit, dependent upon the source of the cells or tissues from which the extract is made, the particular target RNA sequence, and other factors. As will be noted below, certain macromolecules that bind to polyadenylate may be included in particular screening assays or other methods employing the system and methods described herein when that particular protein or other macromolecule is subject to investigation as described herein.

In a further embodiment of the invention, the cell extract may be partially purified or otherwise manipulated. For example, the cell extract may be partially purified to remove certain components before being placed in the system of the invention, before or after being optionally depleted of macromolecules that bind polyadenylate. For example, certain non-specific factors and/or activities unrelated to of interfering with the methods of the present

invention may be removed from the cell extract. The skilled artisan will recognize for the particular target RNA being investigated hereunder the need for partial purification of the extract and the need for depletion of factors that bind polyadenylate. Furthermore, other components may be added to ensure that the system of the invention recapitulates regulated RNA turnover.

The target RNA sequence in the system of the present invention may be an one of a number of RNA or modified RNA molecules. For example, synthetic RNA may be prepared by solid phase synthesis, or reproduced by in vitro transcription using phage polymerase as is known to the skilled artisan. Naturally occurring RNA may be isolated from cells, tissues, and other biological sources. The RNA may be a messenger RNA (mRNA), a preferred species herein, or RNA-DNA derivatives. Messenger RNA typically comprises a 5' cap and a 3' polyadenylate sequence. Chemically modified RNA, such as RNA modified by phosphothioate moiety(ies), is embraced herein.

The particular RNA, including mRNA, used in the system and methods of the present invention may be selected depending on the particular species of mRNA to be studied. Investigations of mRNA turnover, endogenous modulators of its turnover and exogenously added molecules, particularly small molecules which affect mRNA turnover, have important therapeutic implications in the prophylaxis and treatment of a variety of conditions and diseases. Certain mRNAs are short-lived, such as those of cytokines; others are long-lived, such as globin message. The regulation of mRNA lifetimes for particular proteins and particular cell types may be subject to various adverse effects, from infection to external stimuli, which alter the turnover and hence cellular physiology. In various conditions, altered expression of cellular proteins and cellular phenotypes may be consequences of

altered mRNA turnover. Pharmacological intervention of such altered mRNA turnover, to restore an altered turnover, or the induction of an altered turnover to achieve a benefit to the organism, are achievable based upon the systems and methods described herein. For example, a particular mRNA, such as that of the proinflammatory cytokine TNFα, is selected as a target for identification of small molecule modulators that may decrease the turnover, and this prolong the lifetime, and expression, of this protein by inflammatory cells. Such modulators may provide substantial benefit in the treatment of certain immunological diseases wherein an increased secretion of TNFα is beneficial. Conversely, massive overproduction of TNFα in sepsis, or its adverse effects in rheumatoid arthritis and inflammatory bowel disease may be ameliorated by use of an agent which further increases the turnover ands thus decreases the expression of TNFα by inflammatory cells.

The application of the invention herein to other mRNA species is embraced by the teachings herein. In particular, the methods of the present invention facilitate high throughput screening for the identification of modulators of RNA turnover, to be applied to the treatment or prophylaxis of disease.

One aspect of the system and method of the present invention is monitoring the turnover of the target RNA sequence. This may be achieved by any one or a combination of various methods known to the skilled artisan, one of which is the provision of labeled RNA. The target RNA sequence of the present may be unlabeled, labeled, or a combination. For example, after setting up conditions under which the deadenylation and/or degradation of the unlabeled target RNA sequence occurs, its level may be assessed by any of a number of methods utilizing a labeled probe, such as by hybridization, or by way of UV absorbance, gel electrophoresis followed by specific or nonspecific staining, or using an amplification

system, such as phage polymerase, and then quantitation by a suitable amplification-based technique such as the molecular beacon method. Alternatively, and perhaps more simply, the target mRNA sequence may be labeled, and the extent of intact sequence or degraded RNA fragments readily quantitated. Labels such as a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties. These non-limiting examples are provided for purposes of illustration only.

Furthermore, optionally, an RNA molecule or a portion thereof, such as its poly(A) tail, may be detectably labeled using routine protocols readily known to a skilled artisan. Suitable labels include enzymes, fluorophores (*e.g.*, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu^{3+}, to name a few fluorophores), chromophores, radioisotopes, chelating agents, dyes, colloidal gold, latex particles, ligands (*e.g.*, biotin), and chemiluminescent agents. When a control marker is employed, the same or different labels may be used for the receptor and control marker.

In the instance where a radioactive label, such as the isotopes ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re are used, known currently available counting procedures may be utilized. Particular ribonucleotides bay be prepared using the appropriate isotopes, and the labeled RNA prepared by solid phase synthesis. Alternatively, moieties comprising the isotopes may be covalently bound to the RNA. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art. In a further example, biotin moieties may be incorporated into the RNA by

any number of means. Subsequently, the biotinylated RNA or degradation fragments may be quantitated by an avidin reagent.

Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or applied stimulation, e.g. U.V. light to promote fluorescence. Among examples of colored labels, which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734); dye sole particles such as described by Gribnau et al. (U.S. Patent 4,373,932) and May et al. (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559 and 0 281 327); or dyes encapsulated in liposomes as described by Campbell et al. (U.S. Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a luminescent moiety. In addition to these direct labelling devices, indirect labels comprising enzymes can also be used according to the present invention. Various types of enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, **70**. 419-439, 1980 and in U.S. Patent 4,857,453.

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase. Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

As noted herein, turnover of RNA occurs in two steps: deadenylation, which is not dependent upon the presence of nucleotide triphosphates, and degradation, which is so dependent. The level of nucleoside triphosphates, including ribonucleotide and/or deoxyribonucleotide triphosphates, ATP, UTP, CTP, TTP, and/or GTP, in the cell extract may or may not be sufficient to permit the degradation aspect of RNA turnover to occur. In one embodiment of the present invention, the system described herein additionally comprises exogenously added nucleotide triphosphate, preferably ATP.

It was noted during the development of the present invention that the inclusion of a reaction enhancer resulted in a slight stimulation in the efficiency of RNA degradation. This is likely to be due to its ability to promote macromolecular complex formation in vitro. Therefore, the invention herein optionally includes the use of a reaction enhancer such as a polymer, to stimulate interaction among the components of the system. Non-limiting examples include polyvinyl alcohol, polyvinylpyrrolidone and dextran; polyvinyl alcohol is preferred.

The above-described system which recapitulates in vitro the RNA turnover of preselected RNA sequences has several utilities, in particular, the identification of the role of endogenous factors and exogenous modulators in RNA turnover. The present invention is broadly directed to a method for identifying an agent capable of modulating the stability of a target RNA sequence comprising

(A) preparing the system as described hereinabove;

(B) introducing said agent into said system;

(C) determining the extent of turnover of said target RNA sequence; and

(D) identifying an agent able to modulate the extent of said turnover as capable of modulating the stability of said target RNA sequence.

The above method may additionally comprise added nucleotide triphosphate. preferably ATP, for the purposes described above.

Agents whose activity in modulating RNA turnover may de detected in the aforementioned method include but is not limited to an RNA stability modifying molecule.

As described above, the target RNA sequence may be selected as described above, depending on the particular RNA to be studied. The target RNA may be unlabeled target RNA sequence, labeled target RNA sequence, or the combination thereof. Labels include but are not limited to a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, or a combination of fluorescent and quenching moieties.

The monitoring the extent of turnover of said target RNA sequence comprises determining the extent of degradation of said labeled target RNA, by the methods described above.

In particular, the present method may be directed to identifying agents capable of modulating the stability of a target RNA sequence which increases the stability of the target RNA sequence, or alternatively, decreasing the stability of the RNA sequence.

In a particular embodiment, the agent is capable of modulating the activity of a AU rich element binding protein or a C-rich element, but it is not so limited. Examples of AU rich element binding proteins and C-rich element binding proteins are as described herein.

In a further embodiment of the present invention, a method is provided for identifying an agent capable of modulating the stability of a target RNA sequence in the presence of an exogenously added RNA stability modifier comprising

(a) preparing the system as described hereinabove;

(b) introducing said RNA stability modifier into said system;

(c) introducing said agent into said system;

(d) determining the extent of turnover of said target RNA sequence; and

(e) identifying an agent able to modulate the extent of said turnover as capable of modulating the stability of said target RNA sequence in the presence of said exogenously added RNA stability modifier.

This aspect of the invention is directed to identifying agents, in particular small molecules, capable of affecting the activity of a RNA turnover modulator. As described above, such small molecules may be screened to determine their effect on the RNA stabilizing or destabilizing ability of an endogenous mediator, which is added to the test system. Alternatively, it may be used to identify compounds which agonize or antagonize exogenous agents. The components of the system, including nucleotide triphosphate, the target RNA, labels, are as described above. In one aspect of this embodiment, the RNA stability modifier increases the stability of said target RNA sequence, and in a further embodiment, the agent decreases the stability of said target RNA sequence increased by said RNA stability modifier. In another embodiment, the RNA stability modifier decreases the stability of said target RNA sequence, and in a further embodiment, the agent increases the stability of said target RNA sequence decreased by said RNA stability modifier.

Candidate series of RNA stability modifiers include the AU rich element binding proteins, but the invention is not limited to such factors. Examples of known proteins having such elements in the mRNA, and binding proteins to the elements, are described above, however, the invention is not limited to these examples.

Furthermore, in another embodiment, the macromolecules that bind RNA that are removed from the cell extract in accordance with the aforementioned procedures may be added back to the system herein to investigate their role in RNA turnover as well as the effect of agents, in particular small molecules, on RNA turnover modulated by these macromolecules that bind RNA. This embodiment may be applied to any of the methods described herein. In yet another embodiment, the target RNA may be loaded with a macromolecule that binds RNA prior to addition to the system herein, for the same purposes stated above.

As noted above, the cell extract used in any of the methods described herein may be partially purified.

A method is also provided for identifying an agent capable of modulating the deadenylation of a target RNA sequence comprising

(A) preparing the system of the present invention in the absence of a nucleotide triphosphate;

(B) introducing said agent into said system; and

(C) monitoring the deadenylation of said target RNA sequence in said system.

A further method is provided for identifying an agent capable of modulating the deadenylation and degradation of a target RNA sequence comprising

(A) preparing the system of the present invention in the presence of ATP;

(B) introducing said agent into said system: and

(C) monitoring the deadenylation and degradation of said target RNA sequence in said system.

Method are also provided herein for identifying an agent capable of modulating cell growth or cell differentiation in a mammal comprising determining the ability of said agent to modulate the stability of a target RNA sequence involved in the modulation of cell growth or differentiation, utilizing the aforementioned methods. The agent capable of modulating cell growth or cell differentiation may intervene in cellular transformation, or in immune dysregulation.

A further embodiment of the present invention is directed to a method for identifying, characterizing or isolating an endogenous molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof comprising

(A) providing the system of the present invention as described above:

(B) introducing said protein suspected of participating in the regulation of RNA turnover into said system:

(C) monitoring the stability of said target RNA sequence in said system; and

(D) identifying, characterizing or isolating said endogenous molecule able to modulate said deadenylation or degradation as capable of participating in the deadenylation or degradation of RNA or regulation thereof.

The molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof may be protein or RNA.

In another embodiment of the present invention. a method is provided for identifying an agent capable of modulating the degradation a target RNA sequence in the absence of deadenylation comprising

(A) providing a cell extract in the presence of a nucleotide triphosphate;

(B) introducing said agent into said cell extract: and

(C) monitoring the degradation of said target RNA sequence in said extract.

The present invention is also directed to kits for monitoring the stability of a preselected target RNA sequence under conditions capable of recapitulating regulated RNA turnover. Such kits comprise:

(a) cell extract optionally depleted of activity of proteins that bind polyadenylate:

(b) other reagents: and

(c) directions for use of said kit.

A kit may further comprising nucleotide triphosphates. a reaction enhancer. a target RNA sequence. RNA binding proteins. RNA stability modifiers. or any combination thereof. It will be seen by the skilled artisan that the kits of the invention provide the components for carrying out the various methods disclosed herein. such as identifying agents and endogenous factors that modulate RNA turnover. identifying agents which modulate the RNA turnover activity of various factors involved in RNA turnover. and others. in particular use in the screening of small molecules for identifying potentially useful therapeutic agents for the prophylaxis and/or treatment of various conditions or diseases benefitted by modulating RNA turnover. The kits may be prepared to investigate either RNA deadenylation. RNA

degradation, or both, depending on the components as described above. Furthermore, the cell extract may be partially purified. The kit may include reagents for depleting activity of proteins present in the extract which bind polyadenylate; such reagents, such as polyadenylate, polyadenylate bound to a matrix, an antibody to proteins that bind polyadenylate, and such an antibody bound to a matrix.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE I

ELAV Proteins Stabilize Deadenylated Intermediates in a Novel In Vitro mRNA Deadenylation/Degradation System

Set forth herein is a novel *in vitro* mRNA stability system using Hela cell cytoplasmic S100 extracts and exogenous polyadenylated RNA substrates that reproduces regulated aspects of mRNA decay (turnover). The addition of cold poly(A) competitor RNA activated both a sequence-specific deadenylase activity in the extracts as well as a potent, ATP-dependent ribonucleolytic activity. The rates of both deadenylation and degradation were up-regulated by the presence of a variety of AU-rich elements in the body of substrate RNAs. Competition analyses demonstrated that *trans*-acting factors were required for RNA de-stabilization by AU-rich elements. The ~30 kDa ELAV protein, HuR, specifically bound to RNAs containing an AU-rich element derived from the TNF-α mRNA in the *in vitro* system. Interaction of HuR with AU-rich elements, however, was not associated with RNA

destabilization. Interestingly, recombinant ELAV proteins specifically stabilized deadenylated intermediates generated from the turnover of AU-rich element-containing substrate RNAs. Thus, mammalian ELAV proteins play a role in regulating mRNA stability by influencing the access of degradative enzymes to RNA substrates.

The relative stability of mRNA is an important regulator of gene expression. The half-life of a specific mRNA can play a role in determining both its steady state level of expression, as well as the rate at which its gene product is induced (reviewed in Ross, 1995; Caponigro and Parker, 1996). Furthermore, mutations that affect the stability of mRNAs encoding regulatory factors can promote oncogenic transformation and immune dysregulation (Aghib et al., 1990; Schiavi et al., 1992). In general, many short-lived proteins, including those derived from cytokines and proto-oncogenes, are encoded by short-lived mRNAs. Several mRNAs that encode stable proteins, such as a-globin, have also been shown to have extraordinarily long half-lives (Holcik and Liebhaber, 1997). In addition, surveillance mechanisms that identify and reduce the half-lives of aberrant mRNAs that contain nonsense codon mutations have been described (Maquat, 1995; Jacobson and Peltz, 1996). Therefore, regulation of the half-life of mRNAs can have dramatic consequences on cellular responses and functional outcomes during growth and development.

Through the application of genetics, the mechanisms and factors involved in the turnover of mRNA in *Saccharomyces cerevisiae* are beginning to be identified. Multiple pathways of mRNA turnover are present in yeast, allowing for numerous levels of regulation and fine-tuning of gene expression. One general pathway of mRNA decay involves poly(A) tail shortening followed by decapping and 5'-to-3' exonucleolytic decay (Muhlrad et al., 1994). A second general pathway involves deadenylation followed by 3'-to-5' turnover of the body

of the mRNA (Anderson and Parker, 1998). Endonucleolytic cleavage of some mRNAs has also been demonstrated (Presutti et al., 1995). Finally, another alternative decay pathway that bypasses deadenylation is involved in the translation-dependent degradation of nonsense codon-containing mRNAs (Weng et al., 1997). Several degradation enzymes and regulatory proteins that play a role in mRNA stability in yeast have been identified (Caponigro and Parker, 1996; Weng et al., 1997). Functionally significant interactions between the cap structure and the 3' poly(A) tail of yeast mRNAs have also been described (Tarun and Sachs, 1997). Whether these observations are generally applicable to mammalian cells, however, remains to be established.

In vivo observations are beginning to allow some generalizations concerning major pathways of mRNA turnover in mammalian cells. A poly(A) tail of approximately 200 bases is added to most mRNAs during processing in the nucleus (Colgan and Manley, 1997). The poly(A) tail serves at least two known functions in mRNA stability. First, in association with poly(A) binding proteins (Bernstein et al., 1989; Ford et al., 1997), it protects the mRNA from 3'-to-5' exonucleases. Second, the poly(A) tail serves as an initiation site for the turnover of the mRNA. The poly(A) tail can be progressively shortened throughout the lifetime of a mRNA in the cytoplasm. Controlling the rate of deadenylation appears to be an important regulatory point in mRNA stability (Wilson and Treisman, 1988; Xu et al., 1997). Once the poly(A) tail is shortened to approximately 30-65 bases, the body of the mRNA appears to be degraded in a rapid fashion *in vivo* without the accumulation of discernible intermediates (Chen et al., 1995; Xu et al., 1997). Little is known, however, concerning the enzymes and regulatory components involved in mammalian mRNA turnover.

In addition to the poly(A) tail, several *cis*-acting elements have been shown to play a role in mRNA stability. The 5' terminal cap structure protects the transcript from exonucleases (Furuichi et al., 1977). Several destabilizing elements (Caput et al., 1986; Shyu et al., 1989; Bonnieu et al., 1990; Peng et al., 1996), as well as stabilizing elements (Stefonovic et al., 1997), located in the body of the mRNA have also been identified. One well-characterized element that regulates mRNA stability is an AU-rich sequence (ARE) found in the 3' untranslated region of many short-lived mRNAs (Shaw and Kamen, 1986). These AREs primarily consist of AUUUA repeats or a related nonameric sequence (Lagnado et al., 1994; Zubiaga et al., 1995; Xu et al., 1997) and have been divided into three classes based on sequence characteristics and degradation kinetics (Xu et al., 1997). In general, AREs have been shown to increase the rate of deadenylation and RNA turnover in a translation-independent fashion (Chen et al., 1995; Fan et al., 1997). The underlying mechanism behind ARE function, however, remains to be determined.

Numerous proteins have been described that can bind *in vitro* to AU-rich elements (e.g. Malter, 1989; Vakalopoulou et al., 1991; Bohjanen et al., 1991; Brewer, 1991; Levine et al., 1993; Hamilton et al., 1993; Katz et al., 1994; Nakagawa et al., 1995; Ma et al., 1996), but the exact role of each factor in the process of mRNA turnover remains to be defined. The ELAV family of ARE-binding proteins is evolutionarily conserved and differentially expressed in tissues throughout the development of vertebrates (reviewed in Antic and Keene, 1997). Although ELAV proteins have been found in both the cytoplasm and the nucleus (Gao and Keene, 1996), the most ubiquitously expressed form, HuR, can shuttle between the nucleus and the cytoplasm (Fan and Steitz, 1998; Peng et al., 1998; Atasoy et al, 1998). ELAV proteins play an important role in growth and development, as the Drosophila homolog is genetically essential for development and maintenance of the nervous system

(Campos et al., 1985; Robinow and White, 1988). In addition, mammalian ELAV proteins are induced during differentiation and are distributed in RNP granules along dendrites (Gao and Keene, 1996). Several lines of evidence suggest that ELAV proteins control aspects of post-transcriptional gene expression (Gao and Keene, 1996; Koushika et al., 1996; Myer et al., 1997; Ma et al., 1997; Antic and Keene, 1998). Over-expression of ELAV family members, for example, has been shown to affect accumulation of selected mRNAs (Jain et al., 1997; Levy et al., 1998; Fan and Steitz, 1998; Peng et al., 1998). The precise role of ELAV proteins and other ARE-binding factors, however, remains to be established.

Mechanistic questions in mammalian cells are usually best approached using biochemical systems due to the inherent difficulties with mammalian cells as a genetic system. It has been difficult, however, to establish a versatile *in vitro* system to study mRNA stability and turnover. Based on *in vivo* observations and practical considerations, an optimal *in vitro* system to study the process of mRNA stability should have the following properties: First, the system should be efficient and highly reproducible. Second, minimal amounts (preferably undetectable) of RNA degradation in the system should be due to random degradation by non-specific contaminating ribonucleases. Third, deadenylation should occur before general degradation of the mRNA body is observed. Fourth, degradation of the mRNA body should occur in an apparently highly processive fashion without detectable intermediates. Fifth, regulation of the rate of overall deadenylation and degradation should be observed in a sequence-specific manner. Finally, the system should work on exogenous RNAs to allow ease of experimental manipulation.

Reported herein is the discovery of a new and useful *in vitro* mRNA stability system using cytoplasmic S100 extracts that fulfills all of the criteria listed above and possesses all of the

properties known to be involved in ARE-mediated mRNA turnover. This system has been successfully used to demonstrate a role for the AU-rich element binding proteins of the ELAV family in mRNA stability. These findings indicate that ELAV proteins can affect a default pathway of ARE-mediated degradation by either protecting the mRNA from nuclease attack or by displacing factors that otherwise mark these short-lived transcripts for degradation. This *in vitro* system allows the identification of cellular factors involved in mRNA turnover and help elucidate mechanisms involved in the post-transcriptional regulation of gene expression.

Moreover, the *in vitro* system of the invention has ready applications in high throughput assays to screen libraries of compounds to elucidate which compounds may have applications as pharmaceuticals which can modulate the stability and turnover of RNA transcripts *in vivo*, and thus be used to treat a wide variety of disease or disorders.

i. <u>Development of an *In vitro* System that Deadenylates and Degrades RNA Substrates</u>

The development of an *in vitro* system to study mRNA turnover requires the generation of a convenient source of poly(A)$^+$ RNA substrate and an active cellular extract. In order to obtain substrate RNAs that were both polyadenylated and easy to identify using standard acrylamide gel technology, a novel and versatile ligation-PCR approach that can attach a template encoding a 60 base poly(A) tail to the 3' end of DNA fragments that contain a Hind III site was used, and is described *infra*. In initial studies to develop an *in vitro* RNA stability system, a 60 base poly(A) tail was attached to a 54 base polylinker-derived sequence (Gem-A60). The small size of this polyadenylated transcript made it easy to analyze intermediates in the pathway of RNA turnover on acrylamide gels. Cellular extracts were prepared following a standard cytoplasmic S100 protocol (Dignam et al., 1983) using

hypotonically lysed Hela spinner cells with minor variations as described in the Materials and Methods.

Gem-A60 RNA was incubated in S100 extracts in the presence of ATP. As seen in Fig. 1A (left panel), very little turnover of the Gem-A60 RNA was noted after 60 minutes of incubation. This reproducible slow rate of turnover prompted us to hypothesize that an inhibitor of the deadenylation/degradation process might be present in S100 extracts. This hypothesis was based on several observations. First, previous work with nuclear extracts determined that poly(A) binding proteins were strong inhibitors of a 3'-to-5' exonuclease activity (Ford et al., 1997). Second, the activity of a partially purified mammalian deadenylase preparation was inhibited by high amounts of PABP (Korner and Wahle, 1997). Third, over-expression of PABP in *Xenopus* oocytes inhibits maturation-specific deadenylation (Wormington et al., 1996). In order to test whether excess amounts of poly(A) binding proteins were responsible for inhibiting the deadenylation of Gem-A60 RNA in S100 extracts, increasing amounts of cold poly(A) competitor RNA were added to the reaction mixtures to sequester poly(A) binding proteins. As shown in Fig 1A (right side), the addition of poly(A) competitor activated a degradation activity in the S100 extracts. The Gem-A60 RNA was shortened to a species slightly larger than the size of a deadenylated marker (Gem-A0) and approximately 30% of the input RNA was degraded. Titration experiments performed in coordination with UV cross-linking studies demonstrated that the amount of poly(A) competitor RNA required to activate the S100 extract precisely corresponded with the ability of the competitor to inhibit binding of proteins to the poly(A) tail of the substrate RNA (data not shown). Furthermore, the nucleolytic activities activated by the addition of cold poly(A) RNA as competitor to the S100 extracts were still observable at concentrations

of poly(A) >500 ng. These data suggest that the activated nuclease(s) is highly refractory to competition by poly(A).

The progressive shortening of the Gem-A60 RNA substrate observed upon incubation in S100 extract supplemented with poly(A) competitor RNA was determined to be due to a 3'-to-5', poly(A) tail-specific exonuclease based on the following observations: First, RNA substrates ^{32}P-labeled exclusively at their 5' cap structures were progressively shortened in the system in a similar fashion as uniformly labeled transcripts (compare Figs. 1A and 1B). These data suggest that the shortening of the input RNA occurred in a 3'-to-5' direction. This conclusion was confirmed by separately analyzing the 5' and 3' portions of RNA products from the *in vitro* system by RNAse H digestion prior to gel electrophoresis. As shown in Fig. 1C, the 3' portion of the substrate RNA (which consists primarily of the 60 base poly(A) tail) was clearly being degraded before any turnover of the 5' portion of the transcript was detected. After 9 minutes of incubation, 72% of the 3' fragment containing the poly(A) tail is degraded, while only 19% of the 5' fragment has been turned over. Finally, in order to ascertain whether this 3'-to-5' exonuclease activity was indeed a poly(A)-specific deadenylase, we added 15 bases of non-adenylate sequence onto the 3' end of the Gem-A60 RNA (Gem-A60-15). As seen in Fig. 1D, while the Gem-A60 transcript (which contains a 3' poly(A) tail) is an excellent substrate for the 3' exonuclease activity, the Gem-A60-15 RNA, which has its poly(A) tract internalized 15 bases, was not.

From these data it has been concluded that the addition of poly(A) competitor RNA to an S100 extract activates a deadenylase which is active on exogenous, poly(A)+ substrate RNAs. The *in vitro* system reproduces several aspects of mRNA stability observed *in vivo*. The surprising observation that the deadenylase itself is not apparently inhibited by cold

poly(A) suggests that the native enzyme may not have high affinity for its substrate. The deadenylase activity may contain additional RNA binding activities that anchor it to mRNAs, perhaps as part of a multi-component complex.

ii. RNA turnover in the *in vitro* system is regulated by AU-rich instability elements.

It was determined whether the RNA turnover activities exhibited by the S100 extract system could be influenced or modulated by sequences in the body of the transcript in a specific manner. The relative stability of small polyadenylated RNAs containing either a 54 base polylinker sequence (Gem-A60), a 34 base AU-rich element (ARE) from TNF-α mRNA (ARE-A60), or a 72 base ARE from the c-fos mRNA (Fos-A60) was determined in the *in vitro* stability system. As shown in Figs. 2A and 2B, the turnover of both of the ARE-containing RNAs was dramatically increased compared to the Gem-A60 control transcript. To directly assess whether regulation by AREs was occurring in a sequence-specific fashion, the TNF-α-ARE was extensively mutated as described in Materials and Methods. Similar mutations in AU-rich instability elements were shown previously to greatly increase mRNA half-life *in vivo* (Myer et al., 1997). As seen in Fig 2C, mutations in the ARE reduced the rate and extent of deadenylation /degradation over 3-fold in the *in vitro* system. Thus, RNA turnover in the *in vitro* system can be regulated or modulated by AU-rich instability elements in a sequence-specific fashion.

All of the RNA substrates we have examined above contain a body of approximately 50-70 bases attached to a poly(A) tail. It was then determined whether regulated turnover using larger polyadenylated RNA substrates could be detected in the system of the invention. As shown in Fig. 2D, a polyadenylated 250 base RNA derived from the 3' UTR of the SV40 late mRNA (SV-A60) was deadenylated but inefficiently degraded in the *in vitro* system. Adding

the TNF-α-ARE to the 3` portion of this RNA (SVARE-A60) resulted in an approximate 3.5 fold increase in the rate of turnover. Finally, a nearly full length (~950 base) version of the human GM-CSF mRNA was prepared, as well as one in which the ARE was deleted (GM-CSF(-ARE)). The 3` ends of these transcripts were polyadenylated using yeast poly(A) polymerase (Martin and Keller, 1998). Gel purified RNAs were incubated in the *in vitro* stability system and aliquots were removed at the times indicated. As seen in Fig 2E, the version of the GM-CSF mRNA that contains an ARE was approximately 2.5 fold less stable than GM-CSF(-ARE) in the *in vitro* system. As seen above with other transcripts, the GM-CSF transcripts were also deadenylated in the system. Deadenylation was not observable in Fig 2E due to the lack of resolution of the gel system employed, but can be observed using formaldehyde-agarose gels (data not shown).

iii. Degradation, but not deadenylation, requires ATP

Transcripts with 60 adenylates at the 3` end were observed to undergo both deadenylation and turnover in the *in vitro* system. This is consistent with *in vivo* observations that suggest the poly(A) tail is shortened to about 30-65 bases before mRNA turnover is observed (Xu et al., 1997). Since degradation appeared to begin before the input transcript was completely deadenylated (eg. Fig. 2), it was difficult to quantitatively assess the effects of AU-rich elements on relative deadenylation rates. In order to try uncoupling these processes and accurately evaluate the effect of AREs on deadenylation rates in the *in vitro* system, we surveyed the cofactor requirements that might be unique to either deadenylation or turnover. Both processes were inhibited by the addition of EDTA (data not shown), suggesting a role for divalent cations. Curiously, deadenylation could occur without the addition of ATP/phosphocreatine to the system (Fig. 3A). Degradation, on the other hand, required ATP/phosphocreatine as indicated by the accumulation of deadenylated intermediates in its absence (Fig. 3A, lanes –ATP). By omitting ATP from the reaction, therefore, we were able to evaluate relative deadenylation rates in the presence or absence of an AU-rich instability element. RNAs with physiological length poly(A) tails (150-200 bases) which lack (SV-A150-200) or contain (SVARE-A150-200) an ARE were incubated in the *in vitro*

system and aliquots were analyzed at the times indicated. As seen in Fig. 3B, RNA substrates containing an ARE were deadenylated at an approximately two fold faster rate than RNAs that do not contain the instability element.

In summary, an *in vitro* mRNA stability system has been discovered that acts on exogenous substrates and faithfully reproduces all of the known *in vivo* aspects of turnover. RNAs are first deadenylated prior to degradation of the body of the transcript. Degradation of the body of the mRNA then occurs in an apparently highly processive fashion with no discernible intermediates. Deadenylation and decay rates are increased several fold by the inclusion of an AU-rich instability element. ARE regulation of RNA stability is sequence-specific and highly reproducible, as all three of the AREs we have tested in the *in vitro* system function in a similar fashion. This system should provide a valuable means to elucidate mechanistic aspects of regulated and general mRNA turnover pathways.

iv. The role of ARE binding proteins in the *in vitro* system.

The *in vitro* system described here allows evaluation of the role of ARE-binding proteins in the process of RNA deadenylation/degradation. Several proteins were found to be associated with ARE-containing RNAs in our extracts. As seen in Fig. 4A, a protein of ~30 kDa and a group of ~40 kDa proteins were specifically UV cross-linked to the short ARE-A60 transcript. A species of approximately 70 kDa was also detected when this ARE was inserted into a larger transcript (SVARE-A60; see Fig. 5B). It is possible that this 70 kDa protein was not detected on the ARE-A60 RNA because of the relatively small size of the transcript. Efforts to determine the identity of these cross linked species using available antibodies to known ARE-binding proteins revealed the presence of an ELAV protein. As shown in Fig. 4B, immunoprecipitation assays identified the 30 kDa protein as HuR (a.k.a. HuA), a member of the ELAV protein family that is ubiquitously expressed in all tissues (Good, 1995; Ma et al., 1996; Myer et al., 1997). Antisera against another RNA-binding protein of approximately 30 kDa, hnRNP A1, failed to detect any cross linked protein in our system (Fig. 4B). Two additional antisera were tested in order to identify the 40 kDa band. Antibodies to hnRNP C protein failed to detect any cross linked protein , while antisera to AUF-1 (a.k.a. hnRNP D)(Brewer, 1991) did precipitate a small amount of cross linked 40 kDa protein (data not shown). However, this cross linked product was not competed by increasing amounts of a 34 base synthetic ARE competitor RNA (data not shown). The

significance of this low level of non-specific AUF-1 cross linking in the system is unclear. It was concluded that the 30 kDa species that specifically cross links to the ARE element is HuR, a protein that has been previously suggested to play a role in ARE-mediated mRNA decay (Vakaloloupou et al, 1991; Antic and Keene, 1997; Myer et al., 1997).

Next, it was determined whether the interaction of the cross linked ARE binding proteins with the element was required to mediate instability. Synthetic ribonucleotides containing either a 34 base TNF-α ARE or randomly chosen, non-ARE sequences were used. Synthetic competitor RNAs were added in increasing amounts to the *in vitro* stability system and their effect on RNA turnover was assessed. As seen in Fig. 5A, the ARE competitor RNA completely inhibited deadenylation and degradation at 40 pm, while the non-specific RNA had no effect at similar concentrations. The ARE competitor RNA had a similar effect on the deadenylation/degradation of RNAs whether or not they contained an ARE. Thus, factors capable of interacting with AREs are important for deadenylation, and may be a part of a multi-protein deadenylase/degradation complex.

The ability of the synthetic ARE competitor RNA to block deadenylation was compared with the ability of the RNA to compete for interaction of ARE-binding proteins with the substrate transcript. EDTA was added to cross-linking assays to inhibit RNA turnover and to evaluate the effect of various levels of competitor on cross-linking/label transfer efficiency. As shown in Fig. 5B, all ARE-binding proteins (including HuR protein that could be immunoprecipitated using specific antisera prior to gel electrophoresis as shown in panel C) were specifically competed from the SV-ARE-A60 RNA substrates upon addition of 5 pm of the synthetic RNA competitor. As shown in Fig. 5A, however, 5 pm of synthetic ARE competitor RNA failed to have an appreciable effect on the rate of RNA deadenylation/degradation in the system. Hence, none of the ARE-binding proteins that could be detected by cross-linking appear to be required for deadenylation/degradation in the *in vitro* system.

v. <u>ELAV proteins prevent degradation of deadenylated transcripts in the *in vitro* system</u>

Since the ARE binding proteins we detected by cross-linking do not appear to be required for deadenylation/degradation, they may play a role in transcript stability. Consistent with this

model, recent *in vivo* data suggest that overexpression of Hel-N1 and HuR proteins can stabilize ARE-containing transcripts (Jain et al., 1997; Fan and Steitz, 1998; Peng et al., 1998). A mouse recombinant HuR protein, as well as other members of the ELAV family (Hel-N1 and Hel-N2 [a.k.a. HuB]) were produced as GST fusion proteins and added these to the *in vitro* stability system at a 10:1 molar ratio to substrate RNA. Similar data were obtained using any of the three recombinant ELAV family proteins, and only data with rHel-N1 is shown. As seen in Fig. 6A, rHel-N1 protein failed to affect deadenylation of the SVARE-A60 RNA substrate in the *in vitro* system, but stabilized a deadenylated intermediate. GST alone, or another GST-fusion protein that binds RNA (hnRNP H') had no effect on transcript stability in the *in vitro* system (Fig. 6B). As a result, it was concluded that the ELAV family of RNA binding proteins function to protect deadenylated transcripts from the degradation enzymes.

Next, it was tested whether the RNA substrate must contain an ARE in order for rELAV proteins to stabilize a deadenylated intermediate in the *in vitro* system. ARE-A60 RNA, or an unrelated but similarly sized and polyadenylated transcript, CX-A60, were incubated in the *in vitro* system in the presence or absence of rELAV proteins. As seen in Fig. 6C, rHelN1 (or other rELAV proteins [data not shown]) stabilized the deadenylated intermediate only from RNAs that contain an ARE binding site. Thus, the stabilization of deadenylated intermediates by ELAV proteins requires an ARE. Furthermore, ELAV proteins can stabilize a deadenylated intermediate whether the ARE is located at the 3', 5' or central positions of the 250 base SVARE-A60 RNA. These data indicate that the ARE-ELAV protein complex probably is not simply preventing turnover through steric blocking of an end of the transcript, thereby preventing exonuclease access.

Set forth herein is a novel and useful *in vitro* RNA stability system that faithfully reproduces many known aspects of *in vivo* mRNA turnover in mammalian cells. Exogenous RNA substrates are deadenylated before degradation of the RNA body occurs in an apparently highly processive fashion without detectable intermediates. Furthermore, the rates of RNA deadenylation and degradation are regulated by AU-rich elements in the system in a sequence-specific manner. The system of the invention has been successfully used to determine a role for the ELAV family of ARE binding proteins in the stability of

deadenylated transcripts by specifically blocking the degradation step. These data illustrate the value of the system to address the mechanism of regulated mRNA turnover.

The *in vitro* system described in this report has several key technical advantages that significantly increase its utility. First, the system is highly reproducible and uses standard S100 cytoplasmic extracts from Hela spinner cells. In fact, nine independent preparations of S100 extract that all function in the assay in a similar fashion have been tested. The only difference among extracts appears to be in the kinetics of turnover (e.g. compare the slight differences in the pattern of turnover of Gem-A60 RNA in Fig. 1A with the pattern observed in Fig. 1D). Second, the extracts exhibit minimal background degradation of RNA due to non-specific nucleases. This lack of noise in the system significantly contributes to its reproducibility. Another key element of the system is that is uses exogenous polyadenylated RNAs as substrates. This property affords variety in RNA substrate preparation and sequence manipulation. Fourth, the system exhibits sequence-specific regulation by AU-rich elements in the absence of translation. In total, these technical advantages make the system a valuable reagent to identify components involved in mRNA turnover and address the mechanism of regulated mRNA stability.

The addition of poly(A) competitor RNA was required to activate S100 extracts to efficiently deadenylate and degrade RNAs in a regulated manner. Titration of cold poly(A) demonstrated that the extracts became activated for deadenylation/degradation when sufficient competitor was added to substantially reduce cross linking of a 70 kDa poly(A) binding protein to the poly(A) tail of the radiolabeled substrate RNA (data not shown).

Surprisingly, the deadenylation in the extracts remain active even in the presence of >500 ng of poly(A). Commercial poly(A) preparations prepared with polynucleotide phosphorylase, therefore, do not appear to be able to interact with and sequester the deadenylase enzyme. These data suggest that the deadenylase activity is either in extraordinary concentrations in the extracts or may not have a strong affinity for its substrate. In conjunction with this, it has been observed that an increase in deadenylation rate of ARE containing RNAs (Figs. 2 and 3), as well as the ability of the ARE competitor RNA to inhibit deadenylation of non-ARE containing substrates. These data suggest that ARE-binding proteins may be associated with the deadenylase activity.

Moreover, HuR protein, a ubiquitously expressed member of the ELAV family of RNA binding proteins (Good. 1995; Ma et al., 1996; Myer et al., 1997; Antic and Keene, 1997), has been identified as one of the major ARE binding factors in the system of the invention. Also, the system of the invention has been successfully used to detect weak binding to AUF-1 (hnRNP D), a protein previously speculated to be involved in regulated mRNA decay *in vitro* (DeMaria and Brewer, 1996). AUF-1, therefore, does not appear to play a significant role in transcript instability in our system. ELAV proteins are not required for deadenylation/degradation, but rather play a role in the stability of deadenylated RNAs that contain an ARE (Fig. 6). These data suggest that in addition to its effect on deadenylation rates (Chen et al., 1995; Xu et al., 1997), the ARE influences the efficiency of turnover of the body of the mRNA. *In vivo* observations (Chen et al., 1995; Xu et al., 1997; Peng et al., 1998) also support the conclusion that ARE influences mRNA degradation rates.

ELAV proteins, therefore, appear to regulate mRNA stability *in vitro*, an observation consistent with *in vivo* transfection studies. The ELAV family comprises four members, three of which are expressed in a tissue or developmental specific manner (reviewed in Antic and Keene, 1997). Tissue-specific ELAV proteins are also localized primarily to the cytoplasm, while the ubiquitous HuR protein is predominantly nuclear and can redistribute to the cytoplasm (Atasoy et al., 1998; Peng et al., 1998; Fan and Steitz, 1998). It has been suggested that differentially expressed ELAV proteins play a role in regulating the stability of both nuclear and cytoplasmic RNA, thereby fine tuning gene expression in specific developmental states (Gao and Keene, 1996; Antic and Keene, 1998).

The competition data shown in Fig. 5 clearly demonstrate that factors associated with the ARE are required for deadenylation/degradation of substrate RNAs. Based on the kinetics of competition, these factors must either be much more abundant than the cross-linkable ARE binding proteins like HuR, or interact with the ARE with a much lower affinity. We favor the latter model, and suggest that these factors are part of a multi-component complex that includes the deadenylase and degradation enzymes. Through multiple cooperative interactions, these weak ARE binding components may allow efficient assembly of the deadenylase/degradation complex on ARE containing transcripts while still allowing the complex to assemble, albeit less effectively, on non-ARE containing RNAs. The RNA

binding components of this proposed complex also may have affinity for other non-ARE instability elements (e.g. Peng et al., 1996).

The observation that endogenous HuR protein in S100 extracts set forth herein can be cross-linked to ARE-containing RNA substrates (Fig. 5) makes it surprising that an ARE can function as a destablizing element in the *in vitro* assay. Since HuR protein is predominantly nuclear, however, only low levels of the protein are likely to be present in our cytoplasmic extracts. This low level of HuR protein is probably unable to efficiently compete with destablizing factors for binding to the ARE. In fact, sequestration of the HuR protein by the addition of low levels of synthetic ARE competitor RNA does lead to an increased rate of turnover of ARE-containing RNAs in the *in vitro* system. As shown in Fig. 5A, the amount of SVARE-A60 RNA remaining after 30 min. in the system in the absence of competitor RNA (lane 0) is approximately 20% greater than when the assay is done in the presence of 5 pm of ARE competitor RNA (lane 5 pm). The removal or sequestration of HuR protein in S100 extracts, therefore, may be necessary in order to observe regulated deadenylation and degradation in some instances.

Materials and Methods

Transcription templates and RNAs

RNAs were produced by *in vitro* transcription using SP6 polymerase (Melton et al., 1984) in the presence of ^{7m}GpppG cap analog and radiolabeled UTP or ATP as indicated. All transcripts were gel purified prior to use. For RNAs labeled exclusively at the 5' cap, transcription reactions were performed in the absence of cap analog and radioactive nucleotides. Capping was then performed using guanyltransferase (BRL) and radiolabled GTP according to the manufacturer's recommendations. The sequence of short RNAs used as substrates in the *in vitro* system is shown in Table 1.

Transcription templates were derived as follows (Please note that all synthetic oligonucleotides used as transcription templates shown below contain a 24 base SP6 promoter fragment at their 5' ends): Gem-A0 RNA was produced from Hind III cut pGem4 (Promega). Gem-A60-15 RNA was produced from the PCR product used to produce Gem-A60 RNA (see below) without removing the primer binding site with Ssp I. Templates

for ARE-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5'-ATTTAGGTGACACTATAGAATACACATTATTTATTATTTATTTATTATTTATTTA TTTA-3' (SEQ ID NO:1) and its appropriate complement. Templates for MT-ARE-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5'-ATTTAGGTGACACTATAGAATACACGTTAGTATTCATTTGTTTACTATTGATTTC TTTA-3' (SEQ ID NO:2) and its appropriate complement. Templates for Fos-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5'-ATTTAGGTGACACTATAGAATACACAAATTTTATTGTGTTTTTAATTTATTTATT AAGATGGATTCTC-3' (SEQ ID NO:3) and its appropriate complement. The template for SV-A0 RNA was Hind III cut pSVL-Gem (Wilusz et al., 1988). Templates for SVARE-A0 RNA were generated by inserting the TNF-α ARE containing oligonucleotide 5'-ATTATTTATTATTTATTTATTATTTATTATTTA (SEQ ID NO:4) and its appropriate complement between the PstI and Hind III sites of pSVL-Gem (located near the 3' end of the RNA). SVARE-A0 RNA was transcribed from Hind III linearized DNA. The template for GM-CSF (+ARE) RNA was EcoRI cut pGM-CSF (Shaw and Kamen, 1986). The template for GM-CSF (-ARE) RNA was NcoI cut pGM-CSF. Templates for CX-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5'-ATTTAGGTGACACTATAGAATACACCCCAACGGGCCCTCCCTCCCCTCCTTGCA CCATCATCGCATCACG (SEQ ID NO:5) and its appropriate complement.

Synthetic RNAs used in competition studies were made by the NJMS Molecular Core Facility and contained the following sequences: ARE: 5'AUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUA (SEQ ID NO:6); Non-specific competitor: 5'-GUCACGUGUCACC (SEQ ID NO:7).

Addition of Poly(A) tails to transcripts

A template for a 60 base poly(A) tail was added to DNA templates using a ligation/PCR protocol have recently been described (Ford et al., 1997). Briefly, all of the templates described above contain a Hind III site that is used to generate the 3' end of the RNA. The synthetic oligonucleotide 5'-AGCTA$_{60}$TATTGAGGTGCTCGAGGT (SEQ ID NO:8) and its appropriate complement were generated, hybridized, and ligated to Hind III cut DNA templates. Ligation products were amplified using an SP6 promoter primer (5'-CATACGATTTAGGTGACACTATAG (SEQ ID NO:9)) and a primer specific for the 3'

end of the ligated oligonucleotide (5'-ACCTCGAGCACCTC (SEQ ID NO:10)). Amplified products were purified on Centricon 100 columns, cut with SspI, and used as templates for SP6 polymerase generate RNAs carrying the 'A60' designation.

Poly(A) polymerase (Amersham) was used to add 150-200 base poly(A) tails onto transcripts. RNAs were incubated with enzyme according to the manufacturer's recommendations on ice for 5-8 min. Following the reaction, RNAs were extracted with phenol-chloroform, ethanol precipitated, and purified on 5% acrylamide gels containing 7M urea to obtain RNAs with the appropriate amount of poly(A) at the 3' end.

S100 extract production

Cytoplasmic extracts were prepared from Hela spinner cells grown in JMEM supplemented with 10% horse serum as described by Dignam et al (1983) with the following two modifications. First, following centrifugation at 100,000 x g for 1 hr, the supernatant was adjusted to 10% glycerol prior to dialysis. Second, dialysis times were shortened to 30 min. Extracts were stored at −80°C.

In vitro RNA deadenylation/degradation system

Typically, approximately 200,000 cpm (~50 fm) of gel purified RNA is used per reaction. In comparative studies, equal molar amounts of transcripts were used. A typical 14.25 μl reaction mixture contains 3.25 μl of 10% polyvinyl alcohol, 1 μl of a 12.5 mM ATP/ 250 mM phosphocreatine mixture, 1 μl of 500 ng/ul poly(A) (Pharmacia), 1 μl of RNA and 8 μl of dialyzed extract. Reactions were incubated at 30° C for the times indicated and stopped by the addition of 400 μl of stop buffer (400 mM NaCl, 25 mM Tris-Cl, pH 7.6, 0.1% SDS). Reaction mixtures were phenol extracted, ethanol precipitated and analyzed on a 5% acrylamide gel containing 7M urea. All quantitation was performed using a Molecular Dynamics Phosphorimager.

Recombinant ELAV proteins (HuR, Hel-N1 and Hel-N2) were made as GST-fusion proteins in *E. coli* and purified using glutathione-sepharose affinity chromatography according to the manufacturer's recommendations (Levine et al, 1993).

RNase H digestion

ARE-A60 RNA, radiolabeled at A residues, was incubated in the *in vitro* stability system for the times indicated. RNA products were phenol extracted and concentrated by ethanol precipitation. The sample was resuspended in a final volume of 30 μl containing 20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 picomoles of the antisense oligonucleotide 5'-AGTTAAATAAAT (SEQ ID NO:11), and 1 unit of RNase H. Reactions were incubated at 37°C for 30 min. and products were analyzed on a 5% acrylamide gel containing 7 M urea.

UV Cross linking and Immunoprecipitations

UV cross linking/label transfer experiments were performed as described previously using a Sylvania G15T8 germicidal light (Wilusz and Shenk, 1988). Cross linking experiments were done in the presence of 25 mM EDTA to inhibit RNA turnover to allow for accurate comparisons between samples. Following digestion with RNAses A, T1 and T2, cross linked proteins were analyzed on 10% acrylamide gels containing SDS.

For immunoprecipitation analysis following UV cross linking and RNAse treatment, 300 μl of RIPA buffer (0.15M NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris-Cl, pH 7.6) was added to samples. Following a brief centrifugation in a microfuge, precleared samples were incubated on ice with antibodies for 1 hr. Antigen-antibody complexes were collected using formalin fixed, washed protein-A positive *S. aureus* cells, washed five times using RIPA buffer, and analyzed on a 10% acrylamide gel containing SDS. Antibodies specific for GRSF (Qian and Wilusz, 1994) and hnRNP A1 (Wilusz and Shenk, 1990) have been described previously. The preparation and characterization of rabbit polyclonal antibodies specific for HuR will be described elsewhere (Atasoy et al., 1998).

Aghib, D.F., Bishop, J.M., Ottolenghi, S., Guerrasio, A., Serra, A., and Saglio, G. 1990. A 3' truncation of myc caused by chromosomal translocation in a human T-cell leukemia increases mRNA stability. Oncogene **5:** 707-711.

Anderson, J.S.J., and Parker, R. 1998. The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SK12 DEVH box protein and 3' to 5' exonuclease of the exosome complex. EMBO J. **17:** 1497-1506.

Antic, D., and Keene, J.D. 1997. Embryonic lethal abnormal visual RNA-binding proteins involved in growth, differentiation, and posttranscriptional gene expression. Am. J. Hum. Genet. **61:** 273-278.

Antic, D., and Keene, J.D. 1998. Messenger ribonucleoprotein complexes containing human ELAV proteins: interactions with cytoskeleton and translational apparatus. J. Cell. Sci. **111:** 183-197.

Atasoy, U., Watson, J., Patel, D., and Keene, J.D. 1998. Ubiquitously-expressed ELAV RNA binding protein, HuA, is upregulated during serum stimulation and T cell activation and undergoes nuclear / cytoplasmic shuttling. J. Cell Sci., In Press.

Bernstein, P., Peltz, S.W., and Ross, J. 1989. The poly(A)-poly(A) binding protein complex is a major determinant of mRNA stability in vitro. Mol. Cell. Biol. **9:** 659-670.

Bonnieu, A., Roux, P., Marty, L., Jeanteur, P., and Piechaczyk 1990. AUUUA motifs are dispensable for rapid degradation of the mouse c-myc mRNA. Oncogene **5:** 1585-1588.

Bohjanen, P.R., Bronislawa, P., June, C.H., Thompson, C.B., and Lindsten, T. 1991. An inducible cytoplasmic factor (AU-B) binds selectively to AUUUA multimers in the 3' untranslated region of lymphokine mRNA. Mol. Cell. Biol. **11:** 3288-3295.

Brewer, G. 1991. An A+U rich element RNA-binding factor regulates c-myc mRNA stability in vitro. Mol. Cell. Biol. **11:** 2460-2466.

Brown, C.E., Tarun Jr., S.Z., Boeck, R., and Sachs, A.B. 1996. PAN3 encodes a subunit of the Pab1p-dependent poly(A) nuclease in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:** 5744-5753.

Campos, A.R., Grossman, D., and White, K. 1985. Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects: a developmental-genetic analysis. J. Neurogenet. **2:**197-218.

Caponigro, G. and Parker, R. 1996. Mechanism and control of mRNA turnover in Saccharomyces cerevisiae. Microbiol. Rev. **60:** 233-249.

Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. 1986. Identification of a common nucleotide sequence in the 3'-untranslated regions of mRNA molecules specifying inflammatory mediators. Proc. Natl. Acad. Sci. USA **83:** 1670-1674.

Chen, C.-Y.A, Xu, N., and Shyu, A.-B. 1995. mRNA decay mediated by two distinct AU-rich elements from c-fos and GM-CSF transcripts: different deadenylation kinetics and uncoupling from translation. Mol. Cell. Biol. **15:** 5777-5788.

Colgan, D.F., and Manley, J.L. 1997. Mechanism and regulation of mRNA polyadenylation. Genes Dev. **11:** 2755-2766.

DeMaria, C.T., and Brewer, G. 1996. AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. J. Biol. Chem. **271:** 12179-12184.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl. Acids Res. **11:** 1475-1489.

Fan, X.C., Myer, V.E., and Steitz, J.A. 1997. AU-rich elements target small nuclear RNAs as well as mRNAs for rapid degradation. Genes Dev. **11:** 2557-2568.

Fan, X.C., and Steitz, J.A. 1998. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. EMBO J. **17:** 3448-3460.

Ford, L.P., Bagga, P.S., and Wilusz, J. 1997. The poly(A) tail inhibits the assembly of a 3'-to-5' exonuclease in an in vitro RNA stability system. Mol. Cell. Biol. **17:** 398-406.

Furuichi,Y., LaFiandra, A., and Shatkin, A.J. 1977. 5'-terminal structure and mRNA stability. Nature **266:** 235-239.

Gao, F.B., and Keene, J.D. 1996. Hel-N1/Hel-N2 proteins are bound to poly(A)+ mRNA in granular RNP structures and are implicated in neuronal differentiation. J. Cell Sci. **109:** 579-589.

Good, P.J. 1995. A conserved family of elav-like genes in vertebrates. Proc. Natl. Acad. Sci. USA. **92:** 4557-4561.

Hamilton, B.J., Nagy, E., Malter, J.S., Arrick, B.A., and Rigby, W.F.C. 1993. Association of heterogeneous nuclear ribonucleoprotein A1 and C proteins with reiterated AUUUA sequences. J. Biol. Chem. **268:** 8881-8887.

Holcik, M., and Liebhaber, S.A. 1997. Four highly stable eukaryotic mRNAs assemble 3' untranslated region RNA-protein complexes sharing cis and trans components. Proc. Natl. Acad. Sci. USA **94:** 2410-2414.

Jain, R.G., Andrews, L.G., McGowan, K.M., Pekala, P.H., and Keene, J.D. 1997. Ectopic expression of Hel-N1, an RNA binding protein, increases glucose transporter (GLUT 1) expression in 3T3-L1 adipocytes. Mol. Cell. Biol. **17:** 954-962.

Jacobson, A., and Peltz, S.W. 1996. Interrelationships between the pathways of mRNA decay and translation in eukaryotic cells. Annu. Rev. Biochem. **65:** 693-740.

Katz, D.A., Theodorakis, N.G., Cleveland, D.W., Lindsten, T., and Thompson, C.B. 1994. AU-A, an RNA binding activity distinct from hnRNP A1, is selective for AUUUA repeats and shuttles between the nucleus and the cytoplasm. Nucl. Acids Res. **22:** 238-246.

Korner, C.G., and Wahle, E. 1997. Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. J. Biol. Chem. **272:** 10448-10456.

Koushika, S.P., Lisbon, M.J., and White, K. 1996. ELAV, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. Curr. Biol. **6:** 1634-1641.

Lagnado, C.A., Brown, C.Y., and Goodall, G.J. 1994. AUUUA is not sufficient to promote poly(A) shortening and degradation of a mRNA: the functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). Mol. Cell. Biol. **14:** 7984-7995.

Levine, T.D., Gao, F., King, P.H., Andrews, L.G., and Keene, J.D. 1993. Hel-N1: an autoimmune RNA-binding protein with specificity for 3' uridylate-rich untranslated regions of growth factor mRNAs. Mol. Cell. Biol. **13:** 3494-3504.

Levy, N.S., Chung, S., Furneaux, H., and Levy, A.P. 1998. Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J. Biol. Chem. **273:** 6417-6423.

Ma, W.-J., Cheng, S., Wright, A., Campbell, C., and Furneaux, H. 1996. Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. J. Biol. Chem. **271:** 8144-8151.

Ma, W.-J., Cjung, S., and Furneaux, H. 1997. The elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA. Nucl. Acids Res. **25:** 3564-3569.

Malter, J.S. 1989. Identification of an AUUUA-specific messenger RNA binding protein. Science **246:** 664-666.

Martin, G., and Keller, W. 1998. Tailing and 3'-end labeling of RNA with yeast poly(A) polymerase and various nucleotides. RNA **4:** 226-230.

Maquat, L.E. 1995. When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. RNA **1:** 453-465.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids Res. **12:** 7035-7056.

Muhlrad, D., Decker, C.J., and Parker, R. 1994. Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5' to 3' digestion of the transcript. Genes Dev. **8:** 855-866.

Myer, V.E., Fan, X.C., and Steitz, J.A. 1997. Identification of HuR as a protein implicated in AUUUA-mediated mRNA decay. EMBO J. **16:** 2130-2139.

Nakagawa, J., Waldner, H., Meyer-Monard, S., Hofsteenge, J., Jeno, P., and Moronoi, C. 1995. AUH, a novel gene encoding an AU-specific RNA binding protein with intrinsic enoyl-CoA hydralase activity. Proc. Natl. Acad. Sci. USA **92:** 2051-2055.

Peng, S. S.-Y., Chen, C.-Y.A., and Shyu, A.-B. 1996. Functional characterization of a non-AUUUA AU-rich element from the c-jun proto-oncogene mRNA: evidence for a novel class of AU-rich elements. Mol. Cell. Biol. **16:** 1490-1499.

Peng, S.-Y., Chen, C.-Y.A., Xu, N., and Shyu, A.-B. 1998. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J. **17:** 3461-3470.

Presutti, C., Villa, T., Hall, D., Pertica, C., and Bozzoni, I. 1995. Identification of the cis-elements mediating the autogenous control of ribosomal protein L2 mRNA stability in yeast. EMBO J. **14:** 4022-4030.

Qian, Z., and Wilusz, J. 1994. GRSF-1: a poly(A)+ mRNA binding protein which interacts with a conserved G-rich element. Nucl. Acids Res. **22:** 2334-2343.

Ross, J. 1995. mRNA stability in mammalian cells. Microbiol. Rev. **59:** 16-95.

Robinow, S., and White, K. 1988. The locus elav of Drosophila melanogaster is expressed in neurons at all developmental stages. Dev. Biol. **126:** 294-303.

Schiavi, S.C., Belasco, J.G., and Greenberg, M.E. 1992. Regulation of proto-oncogene mRNA stability. Biochem. Biophys Acta **1114:** 95-106.

Shaw, G., and Kamen, R. 1986. A conserved AU rich sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46:** 659-667.

Shyu, A.-B., Belasco, J.G., and Greenberg, M.E. 1989. The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes Dev. **3:** 60-72.

Stefanovic, B., Hellerbrand, C., Holcik, M., Briendl, M., Liebhaber, S.A., and Brenner, D.A. 1997. Posttranscriptional regulation of collagen a1(I) mRNA in hepatic stellate cells. Mol. Cell. Biol. **17:** 5201-5209.

Tarun, S.Z., and Sachs, A.B. 1997. Association of the yeast poly(A) tail binding protein with translation initiation factor eif-4G. EMBO J. **15:** 7168-7177.

Vakalopoulou, E., Schaack, J., and Shenk, T. 1991. A 32-kilodalton protein binds to AU-rich domains in the 3' untranslated regions of rapidly degraded mRNAs. Mol. Cell. Biol. **11:** 3355-3364.

Weng, Y., Ruiz-Echevarria, M.J., Zhang, S., Cui, Y., Czaplinski, K., Dinman, J.D., and Peltz, S.W. 1997. Characterization of the nonsense-mediated mRNA decay pathway and its effect on modulating translation termination and programmed frameshifting. In "mRNA Metabolism and Post-Transcriptional Gene Regulation", J. B. Harford, D.R. Morris, eds., Wiley-Liss, Inc., New York, pp. 241-263.

Wilson, T., and Treisman, R. 1988. Removal of poly(A) tail and consequent degradation of c-fos mRNA facilitated by 3' AU-rich sequence. Nature **366:** 396-399.

Wilusz, J., and Shenk, T. 1988. A 64 kDa nuclear protein binds to RNA segments that include the AAUAAA polyadenylation motif. Cell **52:** 221-228.

Wilusz, J., Feig, D.I., and Shenk, T. 1988. The C proteins of heterogeneous nuclear ribonucleoprotein complexes interact with RNA sequences downstream of polyadenylation cleavage sites. Mol. Cell. Biol. **8:** 4477-4483.

Wilusz, J., and Shenk, T. 1990. A uridylate tract mediates efficient heterogeneous nuclear ribonucleoprotein C protein-RNA cross-linking and functionally substitutes for the downstream element of the polyadenylation signal. Mol. Cell. Biol. **10:** 6397-6407.

Wormington, M., Searfoss, A.M., and Hurney, C.A. 1996. Overexpression of poly(A) binding protein prevents maturation-specific deadenylation and translational inactivation in Xenopus oocytes. EMBO J. **15:** 900-909.

Xu, N., Chen, C.-Y.A., and Shyu, A.-B. 1997. Modulation of the fate of cytoplasmic mRNA by AU-rich elements: key sequence features controlling mRNA deadenylation and decay. Mol. Cell. Biol. **17:** 4611-4621.

Zubiaga, A M., Belasco, J.G., and Greenberg, M.E. 1995. The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. Mol. Cell. Biol. **15:** 2219-2230.

The present invention is not to be limited in scope by the specific embodiments describe herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. An *in vitro* system capable of recapitulating regulated RNA turnover of an exogenously added preselected target RNA sequence comprising a cell extract and said target RNA sequence.

2. The system of claim 1 wherein said regulated RNA turnover is selected from the group consisting of AU-rich element regulated RNA turnover and C-rich element regulated turnover.

3. The system of claim 1 wherein said cell extract is isolated from lysed eukaryotic cells or tissues.

4. The system of claim 3 wherein said cell extract is obtained from a cell line selected from the group consisting of HeLa cells and a T cell line.

5. The system of claim 1 wherein said cell extract is prepared from cells comprising foreign nucleic acid.

6. The system of claim 1 wherein said cell extract is prepared from cells which are infected, stably transfected, or transiently transfected.

7. The system of claim 1 wherein said cell extract is partially purified.

8. The system of claim 1 wherein said cell extract is depleted of activity of proteins that bind polyadenylate.

9. The system of claim 8 wherein said cell extract depleted of activity of proteins that bind polyadenylate is prepared by a method selected from the group consisting of:
   (a) addition to said system of polyadenylate competitor RNA;
   (b) sequestration of proteins that bind polyadenylate;
   (c) addition of a proteinase that inactivates a protein that bind to polyadenylate; and

(d) addition of an agent that prevents the interaction between polyadenylate and an endogenous macromolecule that binds to polyadenylate

10. The system of claim 9 wherein said sequestration of proteins that bind polyadenylate is achieved by treatment of said extract with an material that depletes macromolecules that bind polyadenylate selected from the group consisting of antibodies to proteins that bind polyadenylate, polyadenylate, and the combination thereof.

11. The system of claim 10 wherein said material is attached to a matrix.

12. The system of claim 1 wherein said target RNA sequence is selected from the group of synthetic RNA, naturally occurring RNA, messenger RNA, chemically modified RNA, and RNA-DNA derivatives.

13. The system of claim 12 wherein said target RNA sequence comprises a 5' cap and a 3' polyadenylate sequence.

14. The system of claim 1 wherein said target RNA sequence is selected from the group consisting of unlabeled target RNA sequence, labeled target RNA sequence, and the combination thereof.

15. The system of claim 14 wherein said labeled target RNA sequence is labeled with a moiety is selected from the group consisting of a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties.

16. The system of claim 1 additionally comprising exogenously added nucleotide triphosphate.

17. The system of claim 16 wherein said nucleotide triphosphate is ATP.

18. The system of claim 1 further comprising a reaction enhancer.

19. The system of claim 18 wherein said reaction enhancer is selected from the group consisting of polyvinyl alcohol, polyvinylpyrrolidone and dextran.

20. The system of claim 19 wherein said reaction enhancer is polyvinyl alcohol.

21. A method for identifying an agent capable of modulating the stability of a target RNA sequence comprising
    (A) providine the system of claim 1;
    (B) introducing said agent into said system;
    (C) determining the extent of turnover of said target RNA sequence; and
    (D) identifying an agent able to modulate the extent of said turnover as capable of modulating the stability of said target RNA sequence.

22. The method of claim 21 wherein said system additionally comprises nucleotide triphosphate.

23. The method of claim 22 wherein said nucleotide triphosphate is ATP.

24. The method of claim 21 wherein said agent is an RNA stability modifying molecule.

25. The method of claim 21 wherein said target RNA sequence is selected from the group consisting of unlabeled target RNA sequence, labeled target RNA sequence, and the combination thereof.

26. The method of claim 25 wherein said labeled RNA sequence is labeled with a moiety is selected from the group consisting of a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties.

27. The method of claim 21 wherein said monitoring the extent of turnover of said target RNA sequence comprises determining the extent of degradation of said labeled target RNA.

28. The method of claim 21 wherein said modulating the stability of a target RNA sequence increases the stability of said target RNA sequence.

29. The method of claim 21 wherein said modulating the stability of a target RNA sequence decreases the stability of said RNA sequence.

30. The method of claim 21 wherein said agent is capable of modulating the activity of a AU rich element binding protein or a C-rich element binding protein.

31. The method of claim 30 wherein said AU rich element binding protein is selected from the group consisting of a member of the ELAV protein family; AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B.

32. The method of claim 31 wherein said member of the ELAV protein family is selected from the group consisting of HuR, Hel-N1, HuC and HuD.

33. A method for identifying an agent capable of modulating the stability of a target RNA sequence in the presence of an exogenously added RNA stability modifier comprising

(a) providing the system of claim 1;
(b) introducing said RNA stability modifier into said system;
(c) introducing said agent into said system;
(d) determining the extent of turnover of said target RNA sequence; and
(e) identifying an agent able to modulate the extent of said turnover as capable of modulating the stability of said target RNA sequence in the presence of said exogenously added RNA stability modifier.

34. The method of claim 33 wherein said system additionally comprises nucleotide triphosphate.

35. The method of claim 34 wherein said nucleotide triphosphate is ATP.

36. The method of claim 33 wherein said target RNA sequence is selected from the group consisting of unlabeled target RNA sequence, labeled target RNA sequence, and the combination thereof.

37. The method of claim 36 wherein said labeled RNA sequence is labeled with a moiety is selected from the group consisting of a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties.

38. The method of claim 33 wherein said determining the extent of turnover of said target RNA sequence comprises determining the extent of degradation of said labeled target RNA.

39. The method of claim 33 wherein said RNA stability modifier increases the stability of said target RNA sequence.

40. The method of claim 39 wherein said agent decreases the stability of said target RNA sequence increased by said RNA stability modifier.

41. The method of claim 33 wherein said RNA stability modifier decreases the stability of said target RNA sequence.

42. The method of claim 41 wherein said agent increases the stability of said target RNA sequence decreased by said RNA stability modifier.

43. The method of claim 33 wherein said agent is capable of modulating the activity of a AU rich element binding protein or a C-rich element binding protein.

44. The method of claim 43 wherein said AU rich element binding protein is selected from the group consisting of a member of the ELAV protein family; AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B.

45. The method of claim 44 wherein said member of the ELAV protein family is selected from the group consisting of HuR, Hel-N1, HuC and HuD.

46. A method for identifying an agent capable of modulating the deadenylation of a target RNA sequence comprising
   (A) providing the system of claim 1 in the absence of a nucleotide triphosphate;
   (B) introducing said agent into said system;
   (C) monitoring the deadenylation of said target RNA sequence in said system; and
   (D) identifying an agent able to modulate the extent of said deadenylation as capable of modulating the deadenylation of said target RNA sequence.

47. A method for identifying an agent capable of modulating the deadenylation and degradation of a target RNA sequence comprising
   (A) providing the system of claim 1 in the presence of a nucleotide triphosphate;
   (B) introducing said agent into said system;
   (C) monitoring the deadenylation and degradation of said target RNA sequence in said system; and
   (D) identifying an agent able to modulate the extent of said deadenylation and degradation as capable of modulating the deadenylation and degradation of said target RNA sequence.

48. A method for identifying an agent capable of modulating cell growth or cell differentiation in a mammal comprising determining the ability of said agent to modulate the stability of a target RNA sequence involved in the modulation of cell growth or differentiation in accordance with claim 19.

49. The method of claim 48 wherein said agent capable of modulating cell growth or cell differentiation intervenes in cellular transformation.

50. The method of claim 48 wherein said agent capable of modulating cell growth or cell differentiation intervenes in immune dysregulation.

51. A method for identifying, characterizing or isolating an endogenous molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof comprising

(A) providing the system of claim 1;

(B) introducing said protein suspected of participating in the regulation of RNA turnover into said system;

(C) monitoring the stability of said target RNA sequence in said system; and

(D) identifying, characterizing or isolating said endogenous molecule able to modulate said deadenylation or degradation as capable of participating in the deadenylation or degradation of RNA or regulation thereof.

52. The method of claim 51 wherein said molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof is protein or RNA.

53. A kit for monitoring the stability of a preselected target RNA sequence under conditions capable of recapitulating regulated RNA turnover, said kit comprising:

(a) cell extract depleted of activity of proteins that bind polyadenylate;

(b) other reagents: and

(c) directions for use of said kit.

54. The kit of claim 53 further comprising nucleotide triphosphates, a reaction enhancer, a target RNA sequence, or any combination thereof.

55. A method for identifying an agent capable of modulating the degradation a target RNA sequence in the absence of deadenylation comprising

(A) providing a cell extract in the presence of a nucleotide triphosphate;

(B) introducing said agent into said cell extract: and

(C) monitoring the degradation of said target RNA sequence in said extract.

FIGURE 1 A-D

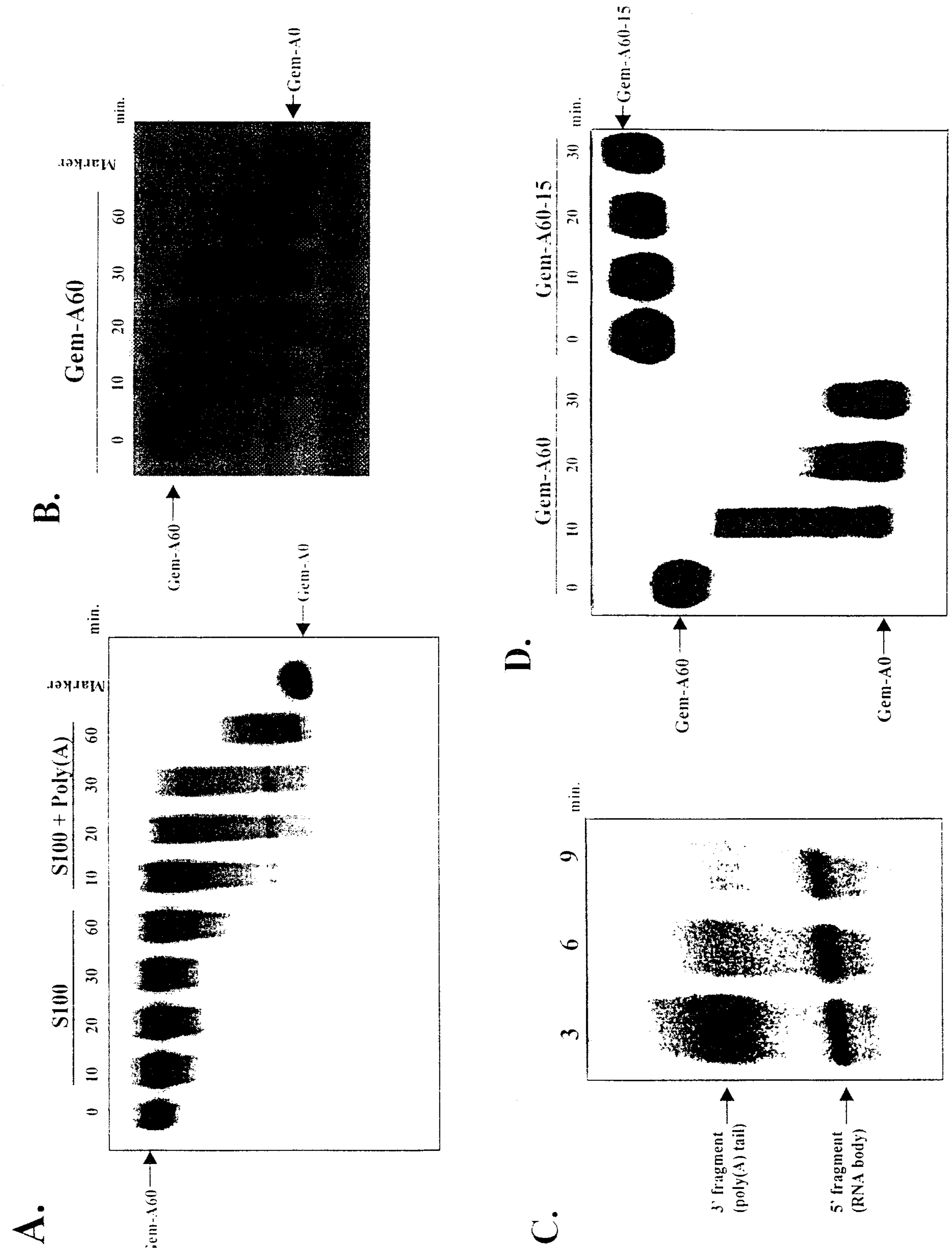

A.
S100
S100 + Poly(A)
Marker
min.
0
10
20
30
60
Gem-A60
Gem-A0
B.
Gem-A60
C.
3
6
9
3' fragment (poly(A) tail)
5' fragment (RNA body)
D.
Gem-A60-15

FIGURE 2 A-B

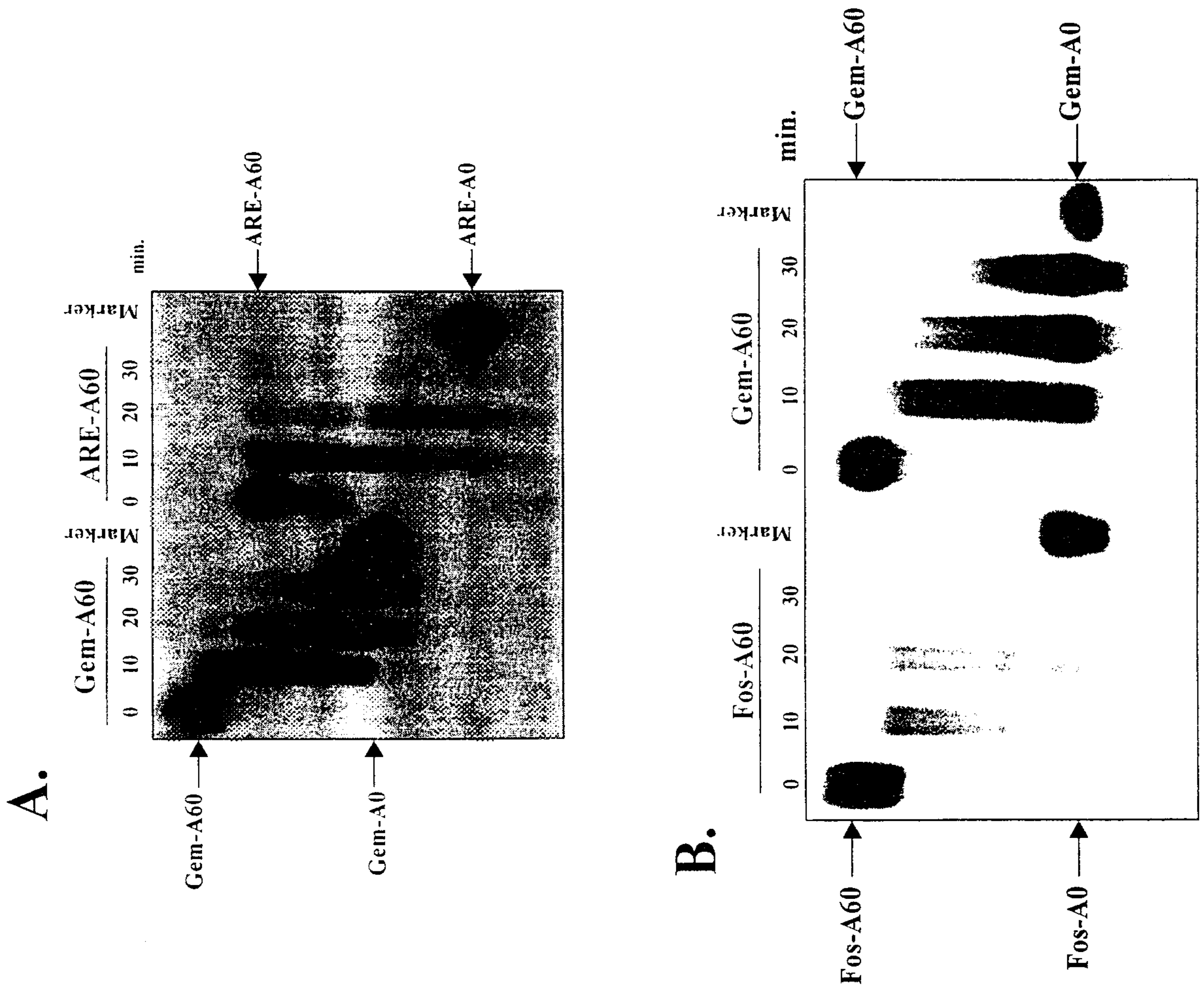

A.
Gem-A60
Gem-A0
Gem-A60
ARE-A60
0 10 20 30
Marker
min.
ARE-A60
ARE-A0
B.
Fos-A60
Fos-A0
Fos-A60
Gem-A60
0 10 20 30
Marker
min.
Gem-A60
Gem-A0

FIGURE 2C

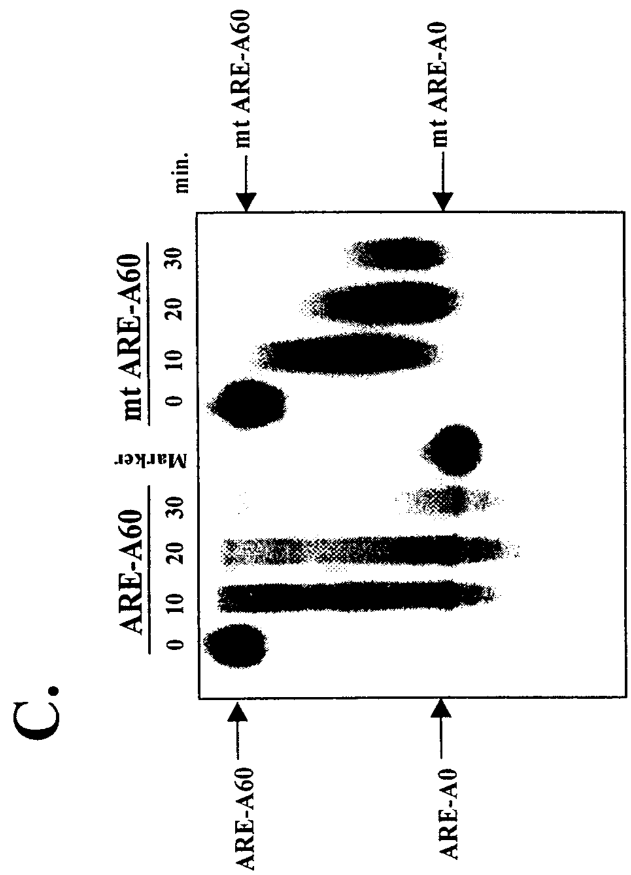

C.
ARE-A60
0
10
20
30
Marker
mt ARE-A60
0
10
20
30
min.
ARE-A60
ARE-A0
mt ARE-A60
mt ARE-A0

FIGURE 2 D-E

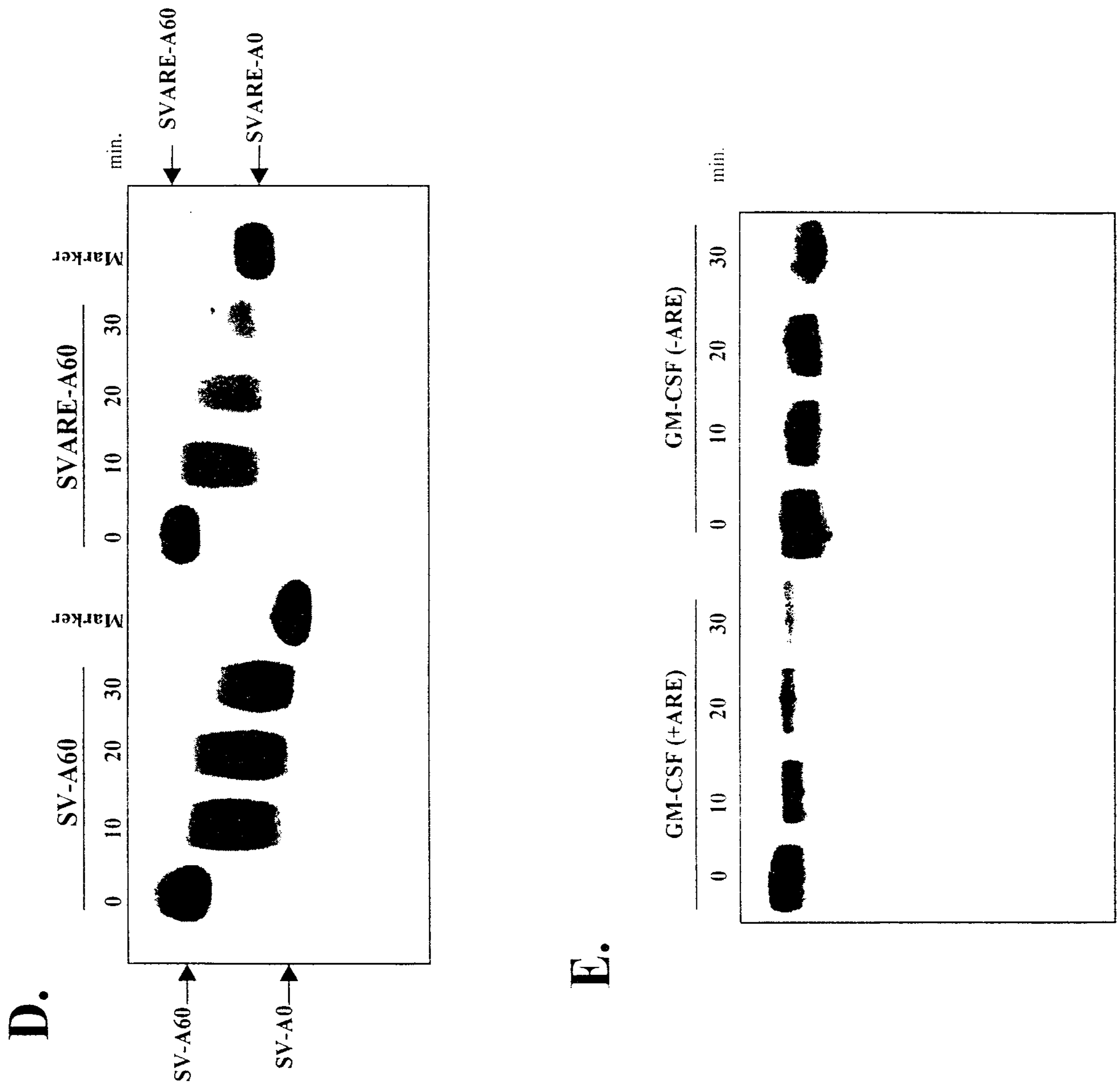
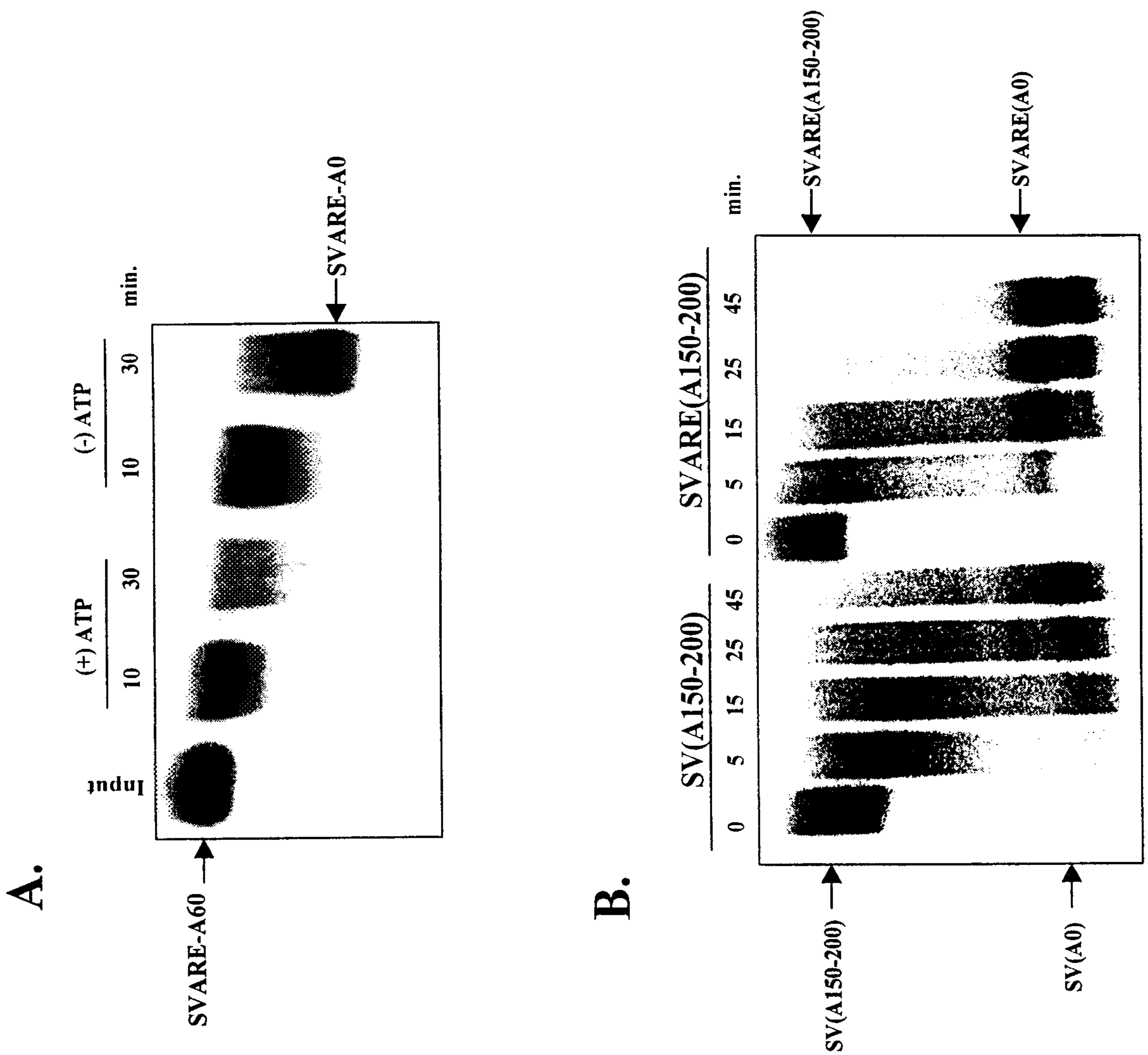

D.
SV-A60
SVARE-A60
Marker
min.
0
10
20
30
SVARE-A60
SVARE-A0
SV-A60
SV-A0
E.
GM-CSF (+ARE)
GM-CSF (-ARE)
min.
0
10
20
30

FIG 3 A-B


A.
SVARE-A60
Input
(+) ATP
10
30
(-) ATP
10
30
min.
SVARE-A0
B.
SV(A150-200)
SV(A0)
SV(A150-200)
0
5
15
25
45
SVARE(A150-200)
0
5
15
25
45
min.
SVARE(A150-200)
SVARE(A0)

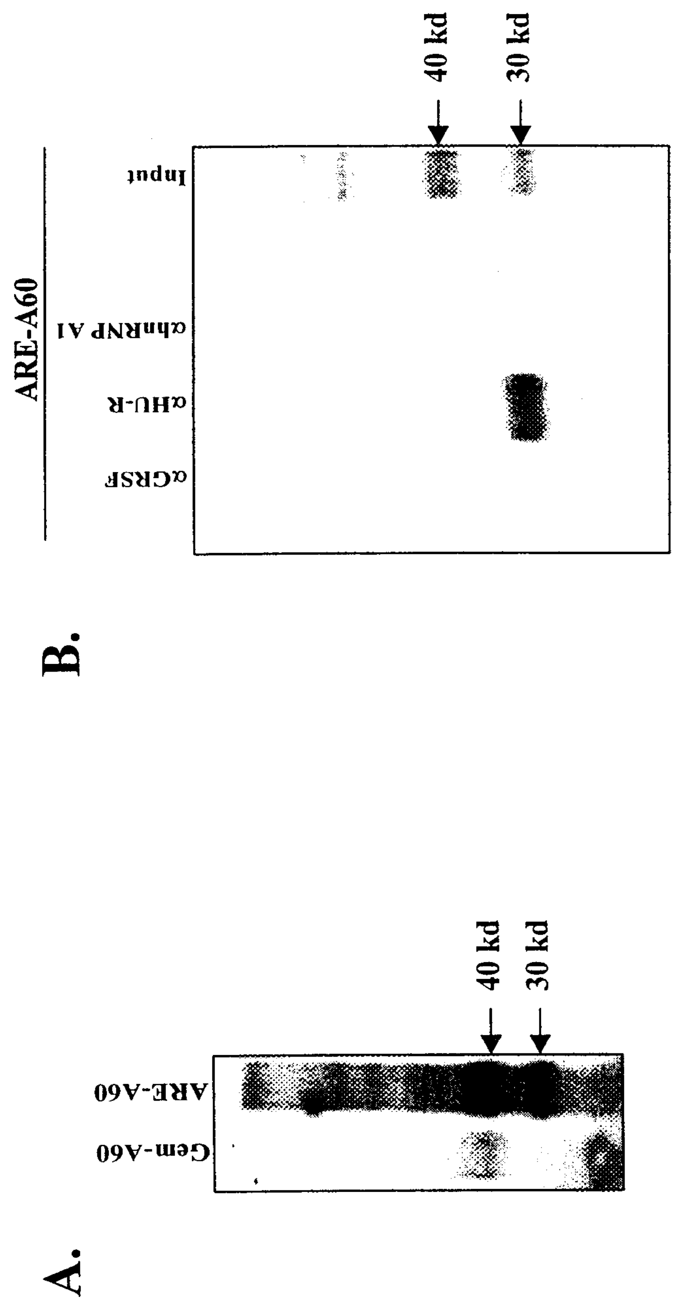

B.
ARE-A60
Input
αhnRNP A1
αHU-R
αGRSF
40 kd
30 kd
A.
ARE-A60
Gem-A60
40 kd
30 kd

FIG 4 A-B

FIG. 5 A-C

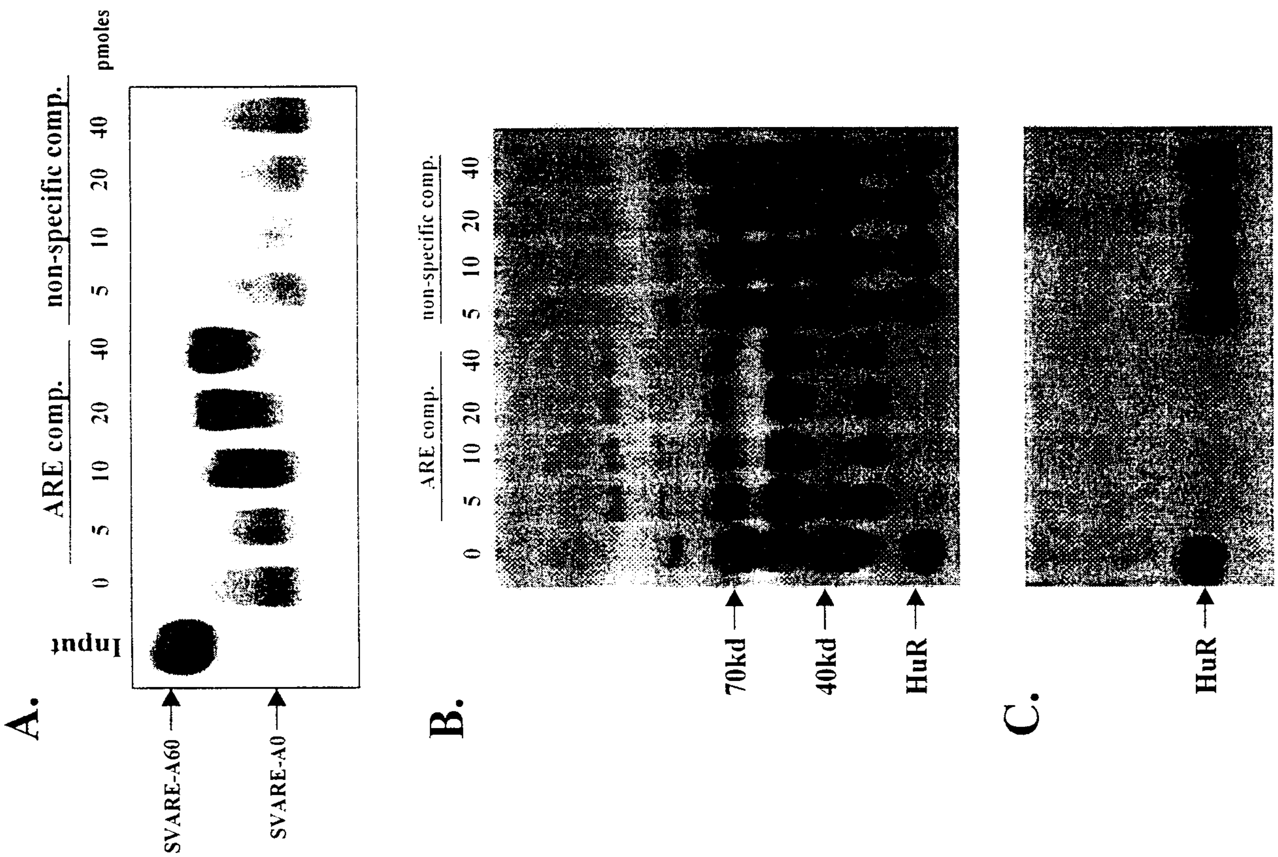
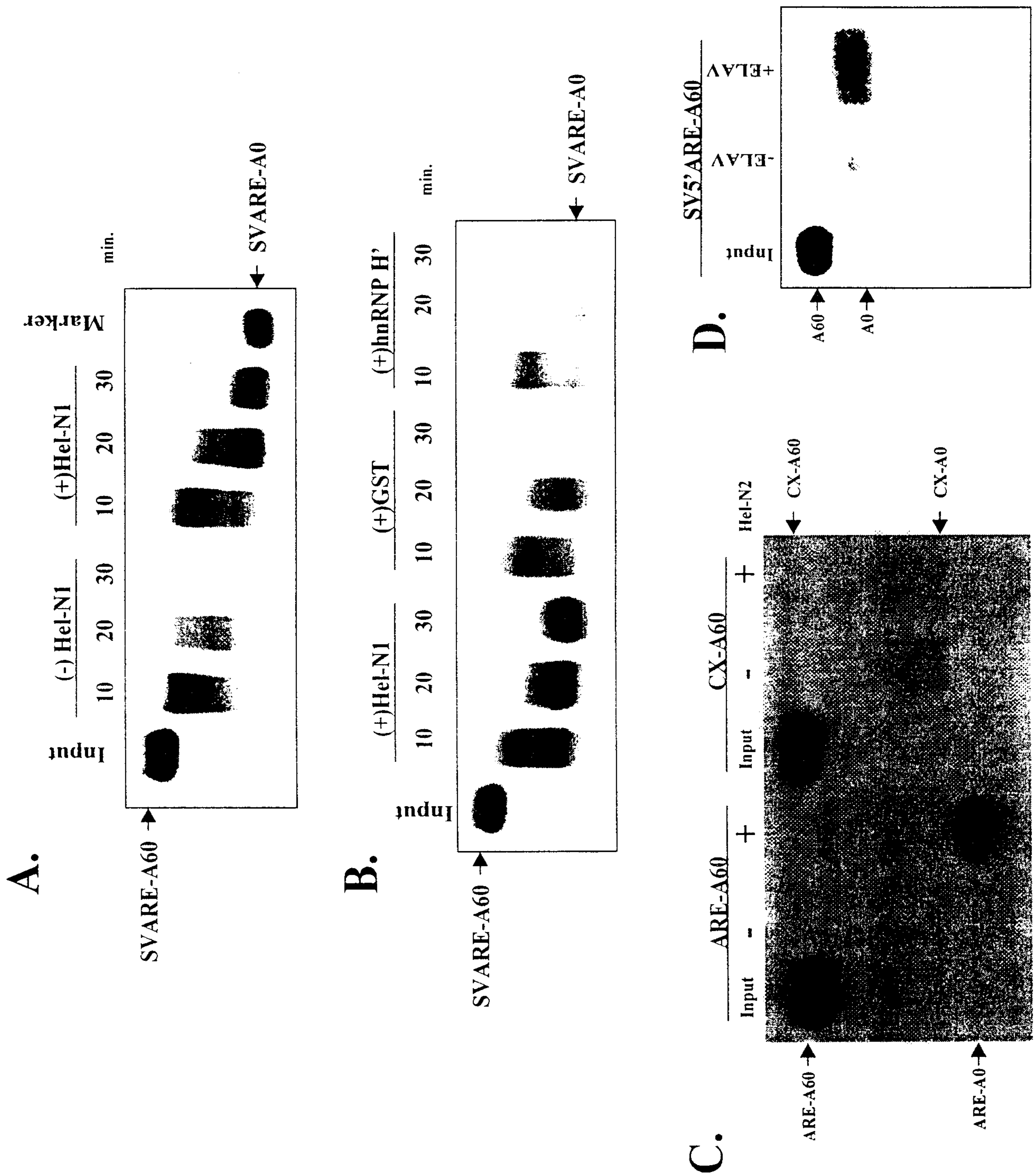

A.
Input
ARE comp.
non-specific comp.
0 5 10 20 40 5 10 20 40
pmoles
SVARE-A60
SVARE-A0
B.
ARE comp.
non-specific comp.
0 5 10 20 40 5 10 20 40
70kd
40kd
HuR
C.
HuR

FIG 6 A-D


A.
Input
(-) Hel-N1
10 20 30
(+)Hel-N1
10 20 30
Marker
min.
SVARE-A60
SVARE-A0
B.
Input
(+)Hel-N1
10 20 30
(+)GST
10 20 30
(+)hnRNP H'
10 20 30
min.
SVARE-A60
SVARE-A0
C.
ARE-A60
Input - +
CX-A60
Input - +
Hel-N2
ARE-A60
ARE-A0
CX-A60
CX-A0
D.
SV5'ARE-A60
Input -ELAV +ELAV
A60
A0

SEQUENCE IDS: 601-1-088

5'-ATTTAGGTGACACTATAGAATACACATTATTTATTATTTATTTATTATTTATTT ATTTA-3' (SEQ ID NO:1)

5'-ATTTAGGTGACACTATAGAATACACGTTAGTATTCATTTGTTTACTATTGATTT CTTTA-3' (SEQ ID NO:2)

5'-ATTTAGGTGACACTATAGAATACACAAATTTTATTGTGTTTTTAATTTATTTAT TAAGATGGATTCTC-3' (SEQ ID NO:3)

5'-ATTATTTATTATTTATTTATTATTTATTATTTA (SEQ ID NO:4)

5'-ATTTAGGTGACACTATAGAATACACCCCAACGGGCCCTCCCTCCCCTCCTTGC ACCATCATCGCATCACG (SEQ ID NO:5)

5'AUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUA (SEQ ID NO:6)

5'-GUCACGUGUCACC (SEQ ID NO:7).

5'-AGCTA$_{60}$TATTGAGGTGCTCGAGGT (SEQ ID NO:8)

5'-CATACGATTTAGGTGACACTATAG (SEQ ID NO:9)

5'-ACCTCGAGCACCTC (SEQ ID NO:10)

5'-AGTTAAATAAAT (SEQ ID NO:11)

AUUUA (SEQ ID NO: 12)

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(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF MEDICINE AND DENTISTRY OF NEW JERSEY** [US/US]; 335 George Street, P.O. Box 2688, New Brunswick, NJ 08903 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **WILUSZ, Jeffrey** [US/US]; 247 Bordentown Avenue, South Amboy, NJ 08879 (US). **FORD, Lance, P.** [US/US]; 30 South Union Avenue, Cranford, NJ 07016 (US).

(74) Agents: **YAMIN, Michael, A.** et al.; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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WO 99/61605 A3

(54) **Title:** SYSTEM FOR REPRODUCING AND MODULATING STABILITY AND TURNOVER OF RNA MOLECULES

(57) **Abstract:** An *in vitro* system is provided that recapitulates regulated mRNA stability and turnover of exogenous RNA substrates. The system comprises a cell extract optionally depleted of activity of proteins that bind polyadenylate, and a target RNA sequence. This system is used for the identification of agents capable of modulating RNA turnover, as well as agents capable of modulating RNA turnover in the presence of RNA stability modifying agents.

INTERNATIONAL SEARCH REPORT

Int 'onal Application No
PCT/US 99/11581

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10 C12Q1/68 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FORD ET AL.: "THE POLY(A) TAIL INHIBITS THE ASSEMBLY OF A 3'-TO 5' EXONUCLEASE IN AN IN VITRO RNA STABILITY SYSTEM" MOL.CELL.BIOL., vol. 17, no. 1, January 1997 (1997-01), pages 398-406, XP000857790 cited in the application the whole document ---	1-55
X	BERNSTEIN ET AL.: "THE POLY(A)-POLY(A)-BINDING PROTEIN COMPLEX IS A MAJOR DETERMINANT OF mRNA STABILITY IN VITRO" MOL.CELL.BIOL., vol. 9, no. 2, February 1989 (1989-02), pages 659-670, XP000857834 cited in the application the whole document ---	1,3, 7-15,53

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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1999	12/01/2000

Name and mailing address of the ISA	Authorized officer
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A	KRIKORIAN AND READ: "IN VITRO DEGRADATION SYSTEM TO STUDY THE VIRION HOST SHUTOFF FUNCTION OF HERPES SIMPLEX VIRUS" J.VIROL., vol. 65, January 1991 (1991-01), pages 112-122, XP000857796 the whole document ---	
A	WO 95 29244 A (WISCONSIN ALUMNI RES FOUND) 2 November 1995 (1995-11-02) the whole document ---	
A	WO 93 23531 A (UNIV DUKE) 25 November 1993 (1993-11-25) the whole document ---	
A	US 5 733 728 A (BREWER GARY ET AL) 31 March 1998 (1998-03-31) the whole document ---	
P,X	FORD AND WILUSZ: "AN IN VITRO SYSTEM USING HELA CYTOPLASMIC EXTRACTS THAT REPRODUCES REGULATED mRNA STABILITY" METHODS, vol. 17, January 1999 (1999-01), pages 21-27, XP002126103 the whole document -----	1-55

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/11581

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9529244 A	02-11-1995	US 5587300 A	24-12-1996
		AU 2295995 A	16-11-1995
WO 9323531 A	25-11-1993	US 5444149 A	22-08-1995
		AU 4231693 A	13-12-1993
		US 5525495 A	11-06-1996
		US 5698427 A	16-12-1997
		US 5773246 A	30-06-1998
US 5733728 A	31-03-1998	NONE	

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 **:** **C12N 15/10, C12Q 1/68, C12P 19/34**	**A2**	**(11) International Publication Number:** **WO 99/61605** **(43) International Publication Date:** 2 December 1999 (02.12.99)

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WILUSZ, Jeffrey [US/US]; 247 Bordentown Avenue, South Amboy, NJ 08879 (US). FORD, Lance, P. [US/US]; 30 South Union Avenue, Cranford, NJ 07016 (US).

(74) Agents: YAMIN, Michael, A. et al.; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: SYSTEM FOR REPRODUCING AND MODULATING STABILITY AND TURNOVER OF RNA MOLECULES

(57) Abstract

An *in vitro* system is provided that recapitulates regulated mRNA stability and turnover of exogenous RNA substrates. The system comprises a cell extract optionally depleted of activity of proteins that bind polyadenylate, and a target RNA sequence. This system is used for the identification of agents capable of modulating RNA turnover, as well as agents capable of modulating RNA turnover in the presence of RNA stability modifying agents.

*(Referred to in PCT Gazette No. 8/2000, Section II)

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SYSTEM FOR REPRODUCING AND MODULATING STABILITY AND TURNOVER OF RNA MOLECULES

GOVERNMENTAL SUPPORT

The research leading to the present invention was supported, at least in part, by grant No. GM56434 from the National Institutes for Health. Accordingly, the Government may have certain rights in the invention.

FIELD OF THE INVENTION

Broadly, the present invention involves a system and method for monitoring the stability of RNA and identifying agents capable of modulating RNA stability.

BACKGROUND OF THE INVENTION

The relative stability of a mRNA is an important regulator of gene expression. The half-life of a mRNA plays a role in determining both the steady state level of expression as well as the rate of inducibility of a gene product. In general, many short-lived proteins are encoded by short-lived mRNAs. Several mRNAs that encode stable proteins, such as α-globin, have also been shown to have extraordinarily long half-lives. Surveillance mechanisms are also used by the cell to identify and shorten the half-lives of mRNAs that contain nonsense codon mutations. Clearly, changes in the half-life of a mRNA can have dramatic consequences on cellular responses and function.

Little is known about mechanisms of mRNA turnover and stability in mammalian cells, but *in vivo* data are beginning to allow some generalizations about major pathways of mRNA turnover. The mRNA poly(A) tail can be progressively shortened throughout the lifetime of a mRNA in the cytoplasm. Controlling the rate of this deadenylation process appears to be a

target for many factors that regulate mRNA stability. Once the poly(A) tail is shortened to approximately 50-100 bases, the body of the mRNA is degraded in a rapid fashion with no discernible intermediates. The process of translation also influences mRNA stability. Little is known, however, concerning the enzymes and regulatory components involved in mammalian mRNA turnover.

Several cis-acting elements have been shown to play a role in mRNA stability. Terminal (5') cap and 3'-poly(A) structures and associated proteins are likely to protect the transcript from exonucleases. Several destabilizing as well as stabilizing elements located in the body of the mRNA have also been identified. The best characterized instability element is an A-U rich sequence (ARE) found in the 3' untranslated region of many short-lived mRNAs. These AREs primarily consist of AUUUA (SEQ ID NO: 12) repeats or a related nonameric sequence. AREs have been shown to increase the rate of deadenylation and mRNA turnover in a translation-independent fashion. For example, proteins with AU-rich elements include many growth factor and cytokine mRNAs, such as c-fos, c-jun, c-myc TNFα, GMCSF, IL1-15, and IFN-β. Other stability elements include C-rich stabilizing elements, such as are found in the mRNAs of globin, collagen, lipoxygenase, and tyrosine hydroxylase. Still other mRNAs have as yet uncharacterized or poorly characterized sequence elements, for example, that have been identified by deletion analysis, e.g. VEGF mRNA.

Numerous proteins have been described that interact with some specificity with an ARE, but their exact role in the process of mRNA turnover remains to be defined. For example, proteins which bind to the ARE described above include HuR and other ELAv family proteins, such as HuR (also called HuA), Hel-N1 (also called HuB), HuC and HuD; AUF1 (four isoforms); tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B. Many others have not been extensively characterized.

Through the application of genetics, the mechanisms and factors involved in the turnover of mRNA in *Saccharomyces cerevisiae* are beginning to be identified. One

major pathway of mRNA decay involves decapping followed by the action of a 5'-to-3' exonuclease. Evidence has also been obtained for a role for 3'-to-5' exonucleases in an alternative pathway. Functionally significant interactions between the cap structure and the 3' poly(A) tail of yeast mRNAs have also been described. Several factors involved in the translation-dependent pathway of nonsense-codon-mediated decay have also been identified. Whether these observations are generally applicable to mammalian cells, however, remains to be established.

Mechanistic questions in mammalian cells are usually best approached using biochemical systems due to the inherent difficulties with mammalian cells as a genetic system. Thus, efforts have been made to develop *in vitro* systems to study mRNA stability and turnover. However, the presently available *in vitro* systems suffer from numerous limitations. For example, many suffer from poor data quality and a general lack of reproducibility that significantly limits their application. Another key problem is that most of these systems do not faithfully reproduce all aspects of mRNA stability. A significant difficulty in the development of these systems is to differentiate between random, non-specific RNA degradation and true, regulated mRNA turnover. The significance of all previous *in vitro* systems to the true *in vivo* process of mRNA stability, therefore, is unclear. To date, no *in vitro* mRNA stability system has been generally accepted in the field as valid and useful. Other problems that have been uncovered in presently available systems are that they usually involve a complicated extract protocol that is not generally reproducible by other laboratories in the field. Also, presently available systems can only be used to assess the stability of endogenous mRNAs, severely limiting their utility. Finally, the data quality obtained using such systems is highly variable, precluding their use in sensitive screening assays.

Accordingly, there exists a need for an *in vitro* RNA stability system is efficient and highly reproducible, and further, one which produces minimal to undetectable amounts of RNA degradation

A further need exists for an *in vitro* RNA stability system wherein deadenylation of an RNA transcript in the system should occur before general degradation of the mRNA body is observed. Also needed is an *in vitro* RNA stability system wherein degradation of the mRNA body occurs in an apparently highly processive fashion without detectable intermediates, and further, the regulation of the rate of overall deadenylation and degradation should be observed in a sequence-specific manner. Such a system should be applicable to exogenous RNAs and allow ease of experimental manipulation.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

SUMMARY OF THE INVENTION

In accordance with the present invention, an *in vitro* system for modulating the stability and turnover of an RNA molecule is provided which models RNA processing in vivo. Thus, the present invention permits high throughput screening of compounds/macromolecules that modulate the stability of eukaryotic RNAs in order to identify and design drugs to affect the expression of selected transcripts, as well as to aid in the characterization of endogenous proteins and other macromolecules involved in mRNA stability. The in vitro system of the present invention is useful as a diagnostic aid for determining the molecular defect in selective disease alleles; development of *in vitro* mRNA stability systems for other eukaryotic organisms including parasites and fungi which should lead to novel drug discovery; and improving gene delivery systems by using the system to identify factors and RNA sequences that affect RNA stability.

Broadly, the present invention extends to an *in vitro* system capable of recapitulating regulated RNA turnover of an exogenously added preselected target RNA sequence, the system comprising a cell extract and a target RNA sequence. In a non-limiting example of

the system described herein. the regulated RNA turnover is AU-rich element regulated RNA turnover or C-rich element regulated RNA turnover.

The cell extract of the system of the present invention is isolated from lysed eukaryotic cells or tissues; the cell extract may be obtained for example from a cell line, such as HeLa cells or a T cell line, but the invention is not so limited. The cell extract may be prepared from cells comprising foreign nucleic acid, such as those that are infected, stably transfected, or transiently transfected. The cell extract may be partially purified.

In one embodiment of the invention, the cell extract may be depleted of activity of proteins that bind polyadenylate. The depletion of activity of proteins that bind polyadenylate from the cell extract may be achieved by any of a number of methods, for example, the addition to the system of polyadenylate competitor RNA; the sequestration of proteins that bind polyadenylate; the addition of a proteinase that inactivates a protein that bind to polyadenylate; or addition of an agent that prevents the interaction between polyadenylate and an endogenous macromolecule that binds to polyadenylate, to name a few. As further examples of the methods for sequestration of proteins that bind polyadenylate, it may be achieved by such non-limiting procedures as the treatment of the extract with an material that depletes macromolecules that bind polyadenylate, such as antibodies to proteins that bind polyadenylate, polyadenylate, and the combination. The material may be attached to a matrix. Other methods to achieve the depletion of the activity of proteins that bind polyadenylate may be used.

The target RNA sequence used in the system may be, by way of non-limiting examples, synthetic RNA, naturally occurring RNA, messenger RNA, chemically modified RNA, or

RNA-DNA derivatives. The target RNA sequence may have a 5' cap and a 3' polyadenylate sequence. The target RNA sequence may be unlabeled target RNA sequence, labeled target RNA sequence, or a the combination of both. The labeled RNA sequence may be labeled with a moiety such as, but not limited to a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties. Other moieties and means for labeling RNA are embraced herein.

The system of the present invention may additionally include exogenously added nucleotide triphosphate; ATP is preferred. It may also include a reaction enhancer to enhance the interaction between the various components present in the system, for example, polymers such as but not limited to polyvinyl alcohol, polyvinylpyrrolidone and dextran; polyvinyl alcohol is preferred.

The present invention is also directed to a method for identifying agents capable of modulating the stability of a target RNA sequence. The method is carried out by preparing the system described above which includes the cell extract depleted of activity of proteins that bind polyadenylate and the target RNA sequence; introducing into the aforesaid system an agent to be tested; determining the extent of turnover of the target RNA sequence by, for example, determining the extent of degradation of the labeled target RNA; and then identifying an agent which is able to modulate the extent of RNA turnover as capable of modulating the stability of the target RNA sequence.

The method described above may additionally include nucleotide triphosphate, ATP being preferred. The agent to be tested may be, but is not limited to, an RNA stability modifying

molecule. The non-limiting selection of the types of target RNA sequence and the non-limiting types of labels useful for the RNA as described hereinabove.

The method of the present invention is useful for identifying agents which can either increases or decrease the stability of said target RNA sequence. Such agents may be capable of modulating the activity of an RNA binding molecule such as, but not limited to, C-rich element binding proteins and AU rich element binding proteins, examples of the latter including HuR and other ELAv family proteins, such as HuR , Hel-N1, HuC and HuD; AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B. This list is provided as illustrative of the types of molecules that may be evaluated in the present invention, but is by no means limiting.

In a further embodiment of the present invention, a method is provided for identifying an agent that is capable of modulating the stability of a target RNA sequence in the presence of an exogenously added RNA stability modifier or RNA binding macromolecule. Non-limiting examples of such molecules are described above. The method is carried out by preparing the system described above which includes the cell extract can be depleted of activity of proteins that bind polyadenylate and the target RNA sequence; introducing into the aforesaid system the exogenously added RNA stability modifier or binding macromolecule and the agent to be tested; determining the extent of turnover of the target RNA sequence by, for example, determining the extent of degradation of the labeled target RNA; and then identifying an agent able to modulating the extent of the RNA turnover as capable of modulating the stability of the target RNA sequence in the presence of the exogenously added RNA stability modifier.

The non-limiting selection of the components of this method are as described above. The aforementioned method is useful, for example, when the RNA stability modifier decreases the stability of said target RNA sequence, and the agent to be identified increases the stability of the target RNA sequence that is decreased by the RNA stability modifier. In addition, the method is useful when the RNA stability modifier increases the stability of the target RNA sequence, and the agent to be identified decreases the stability of the target RNA sequence that is increased by the RNA stability modifier. Non-limiting examples of RNA stability modifiers include C-rich element binding proteins, and AU rich element binding proteins, examples of AU rich element binding proteins, including HuR and other ELAv family proteins, such as HuR , Hel-N1, HuC and HuD; AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B. This list is provided as illustrative of the types of molecules that may be evaluated in the present invention, but is by no means limiting.

The present invention is further directed to a method for identifying an agent capable of modulating the deadenylation of a target RNA sequence comprising preparing the system described above in the absence of nucleotide triphosphate, such as ATP; introducing an agent into the system; and monitoring the deadenylation of the target RNA sequence. Furthermore, the invention is also directed towards a method for identifying an agent capable of modulating the deadenylation and degradation of a target RNA sequence comprising preparing the system described herein in the presence of ATP; introducing the agent into the system; and monitoring the deadenylation and degradation of the target RNA sequence. These embodiments may also be carried out in the presence of an RNA stability modifier or RNA binding macromolecule to determine the ability of the agent to modulate the effect of the modulator or binding molecule on RNA stability.

It is a further aspect of the present invention to provide a method for identifying an agent capable of modulating cell growth or cell differentiation in a mammal comprising determining the ability of said agent to modulate the stability of a target RNA sequence involved in the modulation of cell growth or differentiation in accordance with the methods described above. The agents capable of modulating cell growth or cell differentiation may intervene in such physiological processes as cellular transformation and immune dysregulation, but the invention is not so limiting.

It is yet a further aspect of the present invention to provide a method for identifying, characterizing and isolating an endogenous molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof comprising preparing the system described hereinabove; introducing a protein suspected of participating in the regulation of RNA turnover into said system; and monitoring the stability of the target RNA sequence. The endogenous molecule suspected of participating in the deadenylation and/or degradation of RNA or regulation may be protein or RNA.

In another embodiment of the invention, a method is provided for identifying an agent capable of modulating the degradation a target RNA sequence in the absence of deadenylation comprising providing a cell extract in the presence of a nucleotide triphosphate; introducing said agent into said cell extract; and monitoring the degradation of said target RNA sequence in said extract.

A further aspect of the present invention is directed to a kit for monitoring the stability of a preselected target RNA sequence under conditions capable of recapitulating regulated RNA turnover. The kit comprises a cell extract that optionally may be depleted of activity of

proteins that bind polyadenylate; other reagents; and directions for use. The kit may further comprise nucleotide triphosphates, a reaction enhancer, or both.

Accordingly, it is an object of the invention to provide a system for modulating the stability and turnover of an RNA molecule *in vitro*, which permits a skilled artisan to study the turnover generally, or deadenylation and degradation specifically, of an RNA transcript, and screen drugs which can modulate the stability and turnover of an RNA transcript. The turnover may be in the absence or presence of exogenously added RNA stability modulators, or permit the study of the role of endogenous molecules in RNA turnover.

It is another embodiment of the invention to provide a kit that a skilled artisan can readily use to modulate the stability and turnover of an RNA molecule *in vitro*, and investigate the aforementioned agents.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG.1 A-D: The addition of poly(A) to cytoplasmic S100 extracts activates specific deadenylase and degradation activities. **Panel A**. Poly(A) competitor RNA activates nucleolytic activities in the extract. A capped, radiolabeled 54 base RNA containing a 60 base poly(A) tail (Gem-A60) was incubated at 30° C with S100 extract in the absence (lanes marked S100) or presence (Lanes marked S100 +Poly(A)) of 500 ng of cold poly(A) RNA as described in Materials and Methods of Example 1 for the times indicated. RNA products

were analyzed on a 5% acrylamide gel containing 7M urea. The position of a deadenylated, 54 base transcript (Gem-A0) is indicated on the right. **Panel B**. The shortening of input transcripts is due to a 3'-to-5' exonuclease. Gem-A60 RNA, labeled exclusively at the 5' cap, was incubated in the *in vitro* mRNA stability system for the times indicated. Reaction products were analyzed on a 5% acrylamide gel containing 7M urea. The position of a deadenylated, 54 base transcript (Gem-A0) is indicated on the right. **Panel C**. An alternative approach also demonstrates that the shortening of input transcripts is due to a 3'-to-5' exonuclease. ARE-A60 RNA, radiolabeled at A residues, was incubated in the *in vitro* stability system for the times indicated. Reaction products were hybridized to a DNA oligo and cleaved into 5' and 3' fragments using RNase H. Fragments were analyzed on a 5% acrylamide gel containing 7M urea. **Panel D**. The 3'-to-5' exonuclease activity is a specific deadenylase. Gem-A60 RNA or a variant that contains 18 extra nucleotides after the poly(A) tract (Gem-A60-15) were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The position of a deadenylated, 54 base transcript (Gem-A0) is indicated on the left. $31\pm11.0\%$ of the input Gem-A60 RNA was deadenylated/degraded in 30 min.

FIG. 2 A-E: The rate of transcript degradation in the *in vitro* system is regulated by AU-rich instability elements in a sequence-specific fashion. **Panel A.** AU-rich elements dramatically increase the rate of turnover in the *in vitro* system. Gem-A60 RNA or a polyadenylated transcript that contains the 34 base AU-rich element from the TNF-α mRNA, were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (Gem-A0 and ARE-A0) are indicated. The ARE-A60 RNA was deadenylated/degraded 6.6 ± 0.4 fold faster than Gem-A60 RNA. **Panel B.** The AU-rich element from c-fos mRNA also functions as an

instability element *in vitro*. Gem-A60 RNA or a transcript that contains the 72 base AU-rich element from the c-fos mRNA (Fos-A60) were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (Gem-A0 and Fos-A0) are indicated. The Fos-A60 RNA was deadenylated/degraded 3.5±0.3 fold faster than Gem-A60 RNA. **Panel C.** The ability of AU-rich elements to mediate transcript instability in the *in vitro* system is sequence-specific. ARE-A60 RNA or a variant that contains a mutation at every fourth position (mt ARE-A60: see Materials and Methods) were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (ARE-A0 and mt ARE-A0) are indicated. Mutations in the ARE reduced the rate of deadenylation/degradation by 3.7±1.4 fold compared to the wild type ARE-A60 transcript. **Panel D.** The TNF-α AU-rich element mediates instability in a heterologous context. A polyadenylated 250 base RNA derived from the SV late transcription unit (SV-A60), or a variant that contains the 34 base AU-rich element from the TNF-α mRNA (SVARE-A60), were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (SV-A0 and SVARE-A0) are indicated. SVARE-A60 RNA was deadenylated/degraded 3.5±0.7 fold faster than SV-A60 RNA. **Panel E.** The AU-rich element derived from the GM-CSF mRNA functions *in vitro* on nearly a full length RNA substrate. A nearly full length version of the GM-CSF mRNA that contained an AU-rich element (GM-CSF(+ARE), or a version in which the AU-rich element was deleted (GM-CSF(-ARE), were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. GM-CSF(+ARE) was deadenylated/degraded 2.8+0.2 fold faster than the GM-CSF(-ARE) transcript.

FIG. 3 A-B: Deadenylation occurs in the absence of ATP and is regulated by AU-rich elements *in vitro*. **Panel A.** Degradation, but not deadenylation, requires ATP. SV-ARE-A60 RNA was incubated in the *in vitro* system in the presence ((+) ATP lanes) or absence ((-) ATP lanes) for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of the deadenylated SVARE-A) transcript is indicated. **Panel B.** AU-rich elements regulate the rate of deadenylation on RNA substrates which carry a physiologic length poly(A) tail. SV RNA or SV-ARE RNA (a variant that contains an AU-rich element) were polyadenylated with yeast poly(A) polymerase and species that contained tails of approximately 150-200 bases were gel purified. These RNAs (SV(A150-200) and SVARE(A150-200) were incubated in the *in vitro* stability system for the times indicated. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts (SV-A0 and SVARE-A0) are indicated. SVARE(A150-200) RNA was deadenylated 2.2+0.3% fold faster than the SV(A150-200) transcript.

Figure 4 A-B: The HuR protein of the ELAV family specifically binds to the TNF-α AU-rich element in the *in vitro* system. **Panel A.** Two proteins specifically interact with the TNF-α AU-rich element. Gem-A60 and ARE-A60 RNAs were radiolabeled at U residues and incubated in the *in vitro* stability system for 5 min. in the presence of EDTA (to block degradation and allow for accurate comparisons). Reaction mixtures were irradiated with UV light, cleaved with RNase A, and protein-RNA complexes were analyzed on a 10% acrylamide gel containing SDS. The approximate sizes of the cross linked proteins indicated on the right were deduced from molecular weight markers. **Panel B.** The 30 kDa protein is HuR. Radiolabeled ARE-A60 RNA was incubated in the *in vitro* RNA stability system and cross-linked to associated proteins as described above. Cross linked proteins were

immunoprecipitated using the indicated antisera prior to analysis on a 10% acrylamide gel containing SDS. The lane marked Input denotes total cross linked proteins prior to immunoprecipitation analysis.

Figure 5 A-C: While AU-rich element binding factors are important to promote RNA deadenylation and degradation, the binding of the HuR protein to AU-rich elements is not associated with AU-rich element-mediated transcript instability. **Panel A.** Competition analysis suggests that AU-rich element binding factors are required for deadenylation and degradation of transcripts. SVARE-A60 RNA was incubated in the *in vitro* stability system for 30 min. in the presence of the indicated amounts of a synthetic RNA competitor that contained the TNF-α AU-rich element (ARE comp.) or a non-specific sequence. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The position of deadenylated SVARE-A0 RNA is indicated. **Panel B.** Reaction mixtures were prepared as described in panel A with the addition of EDTA to inhibit RNA turnover. Protein-RNA interactions were analyzed by UV cross linking analysis and analyzed on a 10% acrylamide gel containing SDS. The positions of AU rich element-specific cross linked species is indicated on the left. **Panel C.** Reactions were prepared exactly as described for Panel B, except samples were immunoprecipitated using a-HuR specific antisera prior to gel electrophoresis.

FIG. 6 A-D: ELAV proteins specifically stabilize deadenylated intermediates in the *in vitro* system. **Panel A.** SVARE-A60 RNA was incubated in the *in vitro* system in the presence (lanes (+) Hel-N1)) or the absence (lanes (-) Hel-N1) of 1ug of recombinant Hel-N1 protein. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The position of deadenylated SVARE-A0 transcript is indicated. **Panel B.** SVARE-A60 RNA was incubated

in the *in vitro* system in the presence of 1ug of recombinant Hel-N1 (lanes (+) Hel-N1), GST only (lanes (+) GST), or an unrelated RNA binding protein hnRNP H' (lanes (+) hnRNP H'). RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The position of deadenylated SVARE-A0 transcript is indicated. **Panel C.** ARE-A60 RNA, or an unrelated transcript that lacked an AU-rich element (CX-A60), were incubated in the *in vitro* stability system for 30min. in the presence (+ lanes) or absence (- lanes) of ~1 ug of Hel-N2 protein. RNA products were analyzed on a 5% acrylamide gel containing 7M urea. The positions of deadenylated transcripts are indicated. **Panel D.** A variant of SV-A60 RNA that contained the TNF-α ARE in the 5' portion of the transcript (SV5'AGE-A60) was incubated in the in vitro system for 50 min in the absence (- lane) or presence (+ lane) of 1 μg of Hel-N2 protein. RNA products were analyzed on a 5% acrylamide gel containing 7 M urea. The positions of imput and deadenylated transcripts are indicated.

DETAILED DESCRIPTION OF THE INVENTION

Numerous terms and phrases are used throughout the instant Specification. The meanings of these terms and phrases are set forth below.

In particular, as used herein "half-life" of an RNA molecule refers to the measurement of the decline in the amount of an RNA molecule to serve as a template for the synthesis of its protein product.

As used herein "turnover" refers to the degradation of an RNA molecule. Turnover comprises deadenylation and degradation.

As used herein a "cap" or"5' cap" or "terminal cap", and be used interchangeable, and refer to a 7-methyl guanosine (7mG) cap chemically conjugated to the most 5' nucleotide of the RNA molecule.

As used herein, the phrase "polyadenylic acid (poly(A)) tail" refers to a string of contiguous adenylic acids (polyadenylate) added post transcriptionally to the 3' end of an RNA molecule, such as mRNA.

As used herein, the term "stability" refers to the maintenance of an RNA molecule so that it can function, and thus retard the degradation process of an RNA molecule.

As used herein, the phrase "a polyadenylic acid competitor nucleic acid oligomer" refers to an oligomer comprising contiguous adenylic acids" which can be added to a system of the invention and sequester proteins that bind poly(A). Thus, the degradation of a particular RNA molecule having a poly(A) tail can be modulated.

Also, as used herein, the phrase "restriction endonuclease" refers to an enzyme that recognizes specific nucleotide sequences in a nucleic acid molecule, and produces a double-stranded break within or near the site. Some restriction enzymes, such as *Eco*RI or *Hind*III produce "complementary tails" on each of fragments produced. These tails are said to be "sticky" because under hybridization conditions they can reanneal with each other. Thus, if two separate nucleic acid molecules share the same restriction site, then both will contain complementary single-stranded tails when treated with the same restriction endonuclease, and can be spliced together forming a recombinant nucleic acid molecule.

Naturally, as used herein, the phrase "restriction endonuclease site" refers to a specific nucleotide sequence that is recognized by a specific restriction endonuclease.

Furthermore, numerous conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art can be readily utilized to practice the instant invention. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control.

A "cassette" refers to a segment of a nucleic acid molelcule, such as DNA or RNA, that can be inserted into a vector at specific restriction sites. The segment of the nucleic acid

molelcule may encode a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. Preferably, the transforming DNA should be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine: "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine: "DNA molecules"), or any phosphoester anologs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

The present invention is based upon Applicant's discovery of a heretofore unknown system for activating regulated turnover of RNA molecules *in vitro* that surprisingly and unexpectedly permits a skilled artisan to study and to modulate the stability and thus the turnover of a RNA molecule *in vitro*. Thus, the new and useful system of the invention permits accurate and faithful reproduction of both general and regulated aspects

deadenylation and degradation of an RNA molecule, also referred to herein as recapitulating regulated RNA turnover, particularly a eukaryotic mRNA transcript. In particular, the new and useful system of the invention permits minimal amounts, preferably undetectable, of mRNA turnover, and further, deadenylation of an RNA molecule occurs in the system prior to degradation of the RNA molecule, which mimics the turnover process of RNA found *in vivo*.

The key to the development of the system and methods utilizing the system are based on the discovery that polyadenylate competitor RNA is capable of sequestering proteins that bind polyadenylate and consequently activating the deadenylase enzyme, inducing RNA turnover. As it was heretofore considered that such proteins that bind polyadenylate may contribute to RNA deadenylation, the present finding that such proteins are, in contrast, stabilizers of RNA, led to the realization that the such proteins are interacting with and inactivating destabilizing mediators in vivo. Thus, the present invention is directed to an *in vitro* system capable of recapitulating regulated RNA turnover of an exogenously added preselected target RNA sequence comprising a cell extract depleted of activity of proteins that bind polyadenylate, and a preselected target RNA sequence. In one particular embodiment, the regulated RNA turnover is that modulated by AU-rich element (ARE) regulated RNA turnover. Examples of mRNAs with AU-rich elements include those of, by way of non-limiting example, c-fos; c-jun; c-myc TNF-α, GMCSF, IL1-15, and IFN-β. As noted above, AU-rich elements are sites for binding of numerous proteins, including the ELAV family of ARE-binding proteins, such as HuR , Hel-N1, HuC and HuD; others include AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B.. In another embodiment, the regulated RNA turnover is that modulated by C-rich element (CRE) regulated RNA turnover, such elements as found in the mRNA of globin

mRNAs, collagen, lipoxygenase, and tyrosine hydroxylase. Another mRNA with an as yet uncharacterized sequence element is that of VEGF. The invention, however, is not so limiting as to the particular elements or binding proteins to these elements involved in the regulation of RNA turnover.

The cell extract of the present invention is prepared from lysed eukaryotic cells or tissues. Various methods known to the skilled artisan may be used to prepare the cell extract. Various sources of cells may be used, including fresh cells and tissues, and cells lines. Such cells may comprise foreign nucleic acid, such as in cells that are infected; or are transiently or stably transfected with a mammalian expression vector, the latter as described in more detail below. For certain purposes, for example to investigate the role of infection, and in particular intracellular infection, on RNA turnover, infected cells may be utilized as the source of the cell extract herein. Cells infected with viruses or other intracellular microorganisms such as *Listeria monocytogenes*, HTLV, herpes simplex virus, and HIV, may be employed for these particular circumstances. Furthermore, prior to preparation of the cell extract, cells may be exposed to certain chemical or other extracellular stimuli, for example, hormones, growth factors, and kinase and phosphatase inhibitors, which may alter RNA turnover, for which subsequent studies as described herein may be used to identify the induction of certain proteins involved in modulating RNA turnover, or for the identification of agents which may counteract adverse RNA turnover modulation induced by such stimuli. As will be noted in more detail below, the methods herein may be used to identify agents which may protect cells by interfering with adverse RNA turnover induced by various sources. The cell extract is preferably free of nuclei and nuclear contents and comprises cytoplasm, but this is not essential unless particular components, such as enzymes or other factors, from nuclei, interfere with the operation of the system. In a typical preparation,

which may be modified without departing from the scope of the invention, cells are grown, harvested, lysed, centrifuged for 100,000 x g for 1 hour, and dialyzed. Glycerol may be added to protect the extract if stored frozen.

As described above, a cell used to prepare the cell extract may comprise foreign DNA. An isolated nucleic acid molecule to placed in a system of the invention can initially be inserted into a cloning vector to produce numerous copies of the molecule. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives, *e.g.*, pGEX vectors, pmal-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the nucleic acid molecule into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the nucleic acid molecule are not present in the cloning vector, the ends of the molecule may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the termini of the nucleic acid molecule; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the nucleic acid molecule are generated. Preferably, the cloned nucleic acid molecule is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both *E.*

coli and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences from the yeast 2μ plasmid.

Naturally, any of the methods previously described for the insertion of an isolated nucleic acid molecule into a cloning vector may be used to construct expression vectors containing a nucleic acid molecule consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Mammalian expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, *e.g.*, any expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, such as pED (*Pst*I, *Sal*I, *Sba*I, *Sma*I, and *Eco*RI cloning site, with the vector expressing both the cloned gene and *DHFR*; *see* Kaufman, *Current Protocols in Molecular Biology*, 16.12 (1991). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (*Hind*III, *Xba*I, *Sma*I, *Sba*I, *Eco*RI, and *Bcl*I cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, such as pREP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive RSV-LTR promoter, hygromycin selectable marker; Invitrogen), pCEP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive hCMV immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (*Kpn*I, *Pvu*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, *Bam*H1 cloning site, inducible metallothionein IIa gene promoter, hygromycin selectable marker: Invitrogen), pREP8 (*Bam*H1, *Xho*I, *Not*I, *Hind*III, *Nhe*I, and *Kpn*I cloning site, RSV-LTR promoter, histidinol

selectable marker: Invitrogen), pREP9 (*Kpn*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, and BamHI cloning site, RSV-LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen). Selectable mammalian expression vectors for use in the invention include pRc/CMV (*Hind*III, *Bst*XI, *Not*I, *Sba*I, and *Apa*I cloning site, G418 selection; Invitrogen), pRc/RSV (*Hind*III, *Spe*I, *Bst*XI, *Not*I, *Xba*I cloning site, G418 selection; Invitrogen), and others. Vaccinia virus mammalian expression vectors (*see*, Kaufman, 1991, *supra*) for use according to the invention include but are not limited to pSC11 (*Sma*I cloning site, TK- and β-gal selection), pMJ601 (*Sal*I, *Sma*I, *Afl*I, *Nar*I, *Bsp*MII, *Bam*HI, *Apa*I, *Nhe*I, *Sac*II, *Kpn*I, and *Hind*III cloning site; TK- and β-gal selection), and pTKgptF1S (*Eco*RI, *Pst*I, *Sal*I, *Acc*I, *Hind*II, *Sba*I, *Bam*HI, and Hpa cloning site, TK or XPRT selection).

Once a particular nucleic acid molecule, such as RNA, is inserted into a vector, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few. In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired.

Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu et al., 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

Cells useful for the preparation described herein include immortalized or partially immortalized cells which can be grown in large amounts under defined conditions, such as HeLa cells and various T-cell cell lines. Other sources include tissues, blood cells, or myeloid cells. Other sources are well within the realm of the present invention.

The cell extract of the system described herein is depleted of activity of proteins that bind polyadenylate. This may be achieved by any one or a combination of methods such as the following. While not being bound by theory, each of these methods either removes the proteins that bind polyadenylate, or inactivate the binding activity. These procedures may be applied to the cell extract as it is used in the methods described herein, or the cell extract may be treated beforehand. For example, a polyadenylate competitor RNA may be added to the cell extract to provide an irrelevant RNA sequence to which the binding proteins may bind, thus clearing the target RNA sequence of such binding proteins. In another embodiment, sequestration of proteins that bind polyadenylate may be performed. Sequestration may be achieved by adding to the cell extract or exposing the cell extract to a material that binds the aforementioned proteins, such as antibodies to proteins that bind polyadenylate, or polyadenylate sequences themselves or macromolecules comprising polyadenylate sequences which serve as binding targets for such proteins. Alternatively or in addition, these protein

binding materials may be bound to a matrix, such as agarose beads, and the cell extract passed through a column of such beads to remove the proteins which bind polyadenylate. The preparation of such beads covalently modified to comprise antibodies or RNA sequences, whether polyadenylate or sequences comprising polyadenylate, are known to the skilled artisan. Another means for reducing or eliminating such activity from the cell extract is by exposure to one or more proteinase known to inactivate a protein that bind to polyadenylate. These proteinases may be added to the extract, or bound to a matrix and exposed to the extract, after which inactivation the beads may be removed. A further means encompasses addition to the extract of an agent that prevents the interaction between polyadenylate and an endogenous macromolecule that binds to polyadenylate. These and other methods embraced by the present invention achieve the desired goal of depleting macromolecules that bind polyadenylate from the cell extract, thus allowing the cell extract in combination with the target RNA sequence to undergo in vivo-like RNA turnover. One or a combination of the aforesaid methods may be employed to reduce the level of such protein to an acceptable limit, dependent upon the source of the cells or tissues from which the extract is made, the particular target RNA sequence, and other factors. As will be noted below, certain macromolecules that bind to polyadenylate may be included in particular screening assays or other methods employing the system and methods described herein when that particular protein or other macromolecule is subject to investigation as described herein.

In a further embodiment of the invention, the cell extract may be partially purified or otherwise manipulated. For example, the cell extract may be partially purified to remove certain components before being placed in the system of the invention, before or after being optionally depleted of macromolecules that bind polyadenylate. For example, certain non-specific factors and/or activities unrelated to of interfering with the methods of the present

invention may be removed from the cell extract. The skilled artisan will recognize for the particular target RNA being investigated hereunder the need for partial purification of the extract and the need for depletion of factors that bind polyadenylate. Furthermore, other components may be added to ensure that the system of the invention recapitulates regulated RNA turnover.

The target RNA sequence in the system of the present invention may be an one of a number of RNA or modified RNA molecules. For example, synthetic RNA may be prepared by solid phase synthesis, or reproduced by in vitro transcription using phage polymerase as is known to the skilled artisan. Naturally occurring RNA may be isolated from cells, tissues, and other biological sources. The RNA may be a messenger RNA (mRNA), a preferred species herein, or RNA-DNA derivatives. Messenger RNA typically comprises a 5' cap and a 3' polyadenylate sequence. Chemically modified RNA, such as RNA modified by phosphothioate moiety(ies), is embraced herein.

The particular RNA, including mRNA, used in the system and methods of the present invention may be selected depending on the particular species of mRNA to be studied. Investigations of mRNA turnover, endogenous modulators of its turnover and exogenously added molecules, particularly small molecules which affect mRNA turnover, have important therapeutic implications in the prophylaxis and treatment of a variety of conditions and diseases. Certain mRNAs are short-lived, such as those of cytokines; others are long-lived, such as globin message. The regulation of mRNA lifetimes for particular proteins and particular cell types may be subject to various adverse effects, from infection to external stimuli, which alter the turnover and hence cellular physiology. In various conditions, altered expression of cellular proteins and cellular phenotypes may be consequences of

altered mRNA turnover. Pharmacological intervention of such altered mRNA turnover, to restore an altered turnover, or the induction of an altered turnover to achieve a benefit to the organism, are achievable based upon the systems and methods described herein. For example, a particular mRNA, such as that of the proinflammatory cytokine TNFα, is selected as a target for identification of small molecule modulators that may decrease the turnover, and this prolong the lifetime, and expression, of this protein by inflammatory cells. Such modulators may provide substantial benefit in the treatment of certain immunological diseases wherein an increased secretion of TNFα is beneficial. Conversely, massive overproduction of TNFα in sepsis, or its adverse effects in rheumatoid arthritis and inflammatory bowel disease may be ameliorated by use of an agent which further increases the turnover ands thus decreases the expression of TNFα by inflammatory cells.

The application of the invention herein to other mRNA species is embraced by the teachings herein. In particular, the methods of the present invention facilitate high throughput screening for the identification of modulators of RNA turnover, to be applied to the treatment or prophylaxis of disease.

One aspect of the system and method of the present invention is monitoring the turnover of the target RNA sequence. This may be achieved by any one or a combination of various methods known to the skilled artisan, one of which is the provision of labeled RNA. The target RNA sequence of the present may be unlabeled, labeled, or a combination. For example, after setting up conditions under which the deadenylation and/or degradation of the unlabeled target RNA sequence occurs, its level may be assessed by any of a number of methods utilizing a labeled probe, such as by hybridization, or by way of UV absorbance, gel electrophoresis followed by specific or nonspecific staining, or using an amplification

system, such as phage polymerase, and then quantitation by a suitable amplification-based technique such as the molecular beacon method. Alternatively, and perhaps more simply, the target mRNA sequence may be labeled, and the extent of intact sequence or degraded RNA fragments readily quantitated. Labels such as a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties. These non-limiting examples are provided for purposes of illustration only.

Furthermore, optionally, an RNA molecule or a portion thereof, such as its poly(A) tail, may be detectably labeled using routine protocols readily known to a skilled artisan. Suitable labels include enzymes, fluorophores (*e.g.*, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu^{3+}, to name a few fluorophores), chromophores, radioisotopes, chelating agents, dyes, colloidal gold, latex particles, ligands (*e.g.*, biotin), and chemiluminescent agents. When a control marker is employed, the same or different labels may be used for the receptor and control marker.

In the instance where a radioactive label, such as the isotopes ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re are used, known currently available counting procedures may be utilized. Particular ribonucleotides bay be prepared using the appropriate isotopes, and the labeled RNA prepared by solid phase synthesis. Alternatively, moieties comprising the isotopes may be covalently bound to the RNA. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art. In a further example, biotin moieties may be incorporated into the RNA by

any number of means. Subsequently, the biotinylated RNA or degradation fragments may be quantitated by an avidin reagent.

Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or applied stimulation, e.g. U.V. light to promote fluorescence. Among examples of colored labels, which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734); dye sole particles such as described by Gribnau et al. (U.S. Patent 4,373,932) and May et al. (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559 and 0 281 327); or dyes encapsulated in liposomes as described by Campbell et al. (U.S. Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a luminescent moiety. In addition to these direct labelling devices, indirect labels comprising enzymes can also be used according to the present invention. Various types of enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, **70**. 419-439, 1980 and in U.S. Patent 4,857,453.

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase. Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

As noted herein, turnover of RNA occurs in two steps: deadenylation, which is not dependent upon the presence of nucleotide triphosphates, and degradation, which is so dependent. The level of nucleoside triphosphates, including ribonucleotide and/or deoxyribonucleotide triphosphates, ATP, UTP, CTP, TTP, and/or GTP, in the cell extract may or may not be sufficient to permit the degradation aspect of RNA turnover to occur. In one embodiment of the present invention, the system described herein additionally comprises exogenously added nucleotide triphosphate, preferably ATP.

It was noted during the development of the present invention that the inclusion of a reaction enhancer resulted in a slight stimulation in the efficiency of RNA degradation. This is likely to be due to its ability to promote macromolecular complex formation in vitro. Therefore, the invention herein optionally includes the use of a reaction enhancer such as a polymer, to stimulate interaction among the components of the system. Non-limiting examples include polyvinyl alcohol, polyvinylpyrrolidone and dextran; polyvinyl alcohol is preferred.

The above-described system which recapitulates in vitro the RNA turnover of preselected RNA sequences has several utilities, in particular, the identification of the role of endogenous factors and exogenous modulators in RNA turnover. The present invention is broadly directed to a method for identifying an agent capable of modulating the stability of a target RNA sequence comprising

(A) preparing the system as described hereinabove;

(B) introducing said agent into said system;

(C) determining the extent of turnover of said target RNA sequence; and

(D) identifying an agent able to modulate the extent of said turnover as capable of modulating the stability of said target RNA sequence.

The above method may additionally comprise added nucleotide triphosphate, preferably ATP, for the purposes described above.

Agents whose activity in modulating RNA turnover may de detected in the aforementioned method include but is not limited to an RNA stability modifying molecule.

As described above, the target RNA sequence may be selected as described above, depending on the particular RNA to be studied. The target RNA may be unlabeled target RNA sequence, labeled target RNA sequence, or the combination thereof. Labels include but are not limited to a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, or a combination of fluorescent and quenching moieties.

The monitoring the extent of turnover of said target RNA sequence comprises determining the extent of degradation of said labeled target RNA, by the methods described above.

In particular, the present method may be directed to identifying agents capable of modulating the stability of a target RNA sequence which increases the stability of the target RNA sequence, or alternatively, decreasing the stability of the RNA sequence.

In a particular embodiment, the agent is capable of modulating the activity of a AU rich element binding protein or a C-rich element, but it is not so limited. Examples of AU rich element binding proteins and C-rich element binding proteins are as described herein.

In a further embodiment of the present invention, a method is provided for identifying an agent capable of modulating the stability of a target RNA sequence in the presence of an exogenously added RNA stability modifier comprising

(a) preparing the system as described hereinabove;

(b) introducing said RNA stability modifier into said system;

(c) introducing said agent into said system;

(d) determining the extent of turnover of said target RNA sequence; and

(e) identifying an agent able to modulate the extent of said turnover as capable of modulating the stability of said target RNA sequence in the presence of said exogenously added RNA stability modifier.

This aspect of the invention is directed to identifying agents, in particular small molecules, capable of affecting the activity of a RNA turnover modulator. As described above, such small molecules may be screened to determine their effect on the RNA stabilizing or destabilizing ability of an endogenous mediator, which is added to the test system. Alternatively, it may be used to identify compounds which agonize or antagonize exogenous agents. The components of the system, including nucleotide triphosphate, the target RNA, labels, are as described above. In one aspect of this embodiment, the RNA stability modifier increases the stability of said target RNA sequence, and in a further embodiment, the agent decreases the stability of said target RNA sequence increased by said RNA stability modifier. In another embodiment, the RNA stability modifier decreases the stability of said target RNA sequence, and in a further embodiment, the agent increases the stability of said target RNA sequence decreased by said RNA stability modifier.

Candidate series of RNA stability modifiers include the AU rich element binding proteins, but the invention is not limited to such factors. Examples of known proteins having such elements in the mRNA, and binding proteins to the elements, are described above, however, the invention is not limited to these examples.

Furthermore, in another embodiment, the macromolecules that bind RNA that are removed from the cell extract in accordance with the aforementioned procedures may be added back to the system herein to investigate their role in RNA turnover as well as the effect of agents, in particular small molecules, on RNA turnover modulated by these macromolecules that bind RNA. This embodiment may be applied to any of the methods described herein. In yet another embodiment, the target RNA may be loaded with a macromolecule that binds RNA prior to addition to the system herein, for the same purposes stated above.

As noted above, the cell extract used in any of the methods described herein may be partially purified.

A method is also provided for identifying an agent capable of modulating the deadenylation of a target RNA sequence comprising

(A) preparing the system of the present invention in the absence of a nucleotide triphosphate;

(B) introducing said agent into said system; and

(C) monitoring the deadenylation of said target RNA sequence in said system.

A further method is provided for identifying an agent capable of modulating the deadenylation and degradation of a target RNA sequence comprising

(A) preparing the system of the present invention in the presence of ATP;

(B) introducing said agent into said system: and

(C) monitoring the deadenylation and degradation of said target RNA sequence in said system.

Method are also provided herein for identifying an agent capable of modulating cell growth or cell differentiation in a mammal comprising determining the ability of said agent to modulate the stability of a target RNA sequence involved in the modulation of cell growth or differentiation, utilizing the aforementioned methods. The agent capable of modulating cell growth or cell differentiation may intervene in cellular transformation, or in immune dysregulation.

A further embodiment of the present invention is directed to a method for identifying, characterizing or isolating an endogenous molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof comprising

(A) providing the system of the present invention as described above;

(B) introducing said protein suspected of participating in the regulation of RNA turnover into said system;

(C) monitoring the stability of said target RNA sequence in said system; and

(D) identifying, characterizing or isolating said endogenous molecule able to modulate said deadenylation or degradation as capable of participating in the deadenylation or degradation of RNA or regulation thereof.

The molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof may be protein or RNA.

In another embodiment of the present invention, a method is provided for identifying an agent capable of modulating the degradation a target RNA sequence in the absence of deadenylation comprising

(A) providing a cell extract in the presence of a nucleotide triphosphate;

(B) introducing said agent into said cell extract; and

(C) monitoring the degradation of said target RNA sequence in said extract.

The present invention is also directed to kits for monitoring the stability of a preselected target RNA sequence under conditions capable of recapitulating regulated RNA turnover. Such kits comprise:

(a) cell extract optionally depleted of activity of proteins that bind polyadenylate;

(b) other reagents; and

(c) directions for use of said kit.

A kit may further comprising nucleotide triphosphates, a reaction enhancer, a target RNA sequence, RNA binding proteins, RNA stability modifiers, or any combination thereof. It will be seen by the skilled artisan that the kits of the invention provide the components for carrying out the various methods disclosed herein, such as identifying agents and endogenous factors that modulate RNA turnover, identifying agents which modulate the RNA turnover activity of various factors involved in RNA turnover, and others, in particular use in the screening of small molecules for identifying potentially useful therapeutic agents for the prophylaxis and/or treatment of various conditions or diseases benefitted by modulating RNA turnover. The kits may be prepared to investigate either RNA deadenylation, RNA

degradation, or both, depending on the components as described above. Furthermore, the cell extract may be partially purified. The kit may include reagents for depleting activity of proteins present in the extract which bind polyadenylate; such reagents, such as polyadenylate, polyadenylate bound to a matrix, an antibody to proteins that bind polyadenylate, and such an antibody bound to a matrix.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE I

ELAV Proteins Stabilize Deadenylated Intermediates in a Novel In Vitro mRNA Deadenylation/Degradation System

Set forth herein is a novel *in vitro* mRNA stability system using Hela cell cytoplasmic S100 extracts and exogenous polyadenylated RNA substrates that reproduces regulated aspects of mRNA decay (turnover). The addition of cold poly(A) competitor RNA activated both a sequence-specific deadenylase activity in the extracts as well as a potent, ATP-dependent ribonucleolytic activity. The rates of both deadenylation and degradation were up-regulated by the presence of a variety of AU-rich elements in the body of substrate RNAs. Competition analyses demonstrated that *trans*-acting factors were required for RNA de-stabilization by AU-rich elements. The ~30 kDa ELAV protein, HuR, specifically bound to RNAs containing an AU-rich element derived from the TNF-α mRNA in the *in vitro* system. Interaction of HuR with AU-rich elements, however, was not associated with RNA

destabilization. Interestingly, recombinant ELAV proteins specifically stabilized deadenylated intermediates generated from the turnover of AU-rich element-containing substrate RNAs. Thus, mammalian ELAV proteins play a role in regulating mRNA stability by influencing the access of degradative enzymes to RNA substrates.

The relative stability of mRNA is an important regulator of gene expression. The half-life of a specific mRNA can play a role in determining both its steady state level of expression, as well as the rate at which its gene product is induced (reviewed in Ross, 1995; Caponigro and Parker, 1996). Furthermore, mutations that affect the stability of mRNAs encoding regulatory factors can promote oncogenic transformation and immune dysregulation (Aghib et al., 1990; Schiavi et al., 1992). In general, many short-lived proteins, including those derived from cytokines and proto-oncogenes, are encoded by short-lived mRNAs. Several mRNAs that encode stable proteins, such as a-globin, have also been shown to have extraordinarily long half-lives (Holcik and Liebhaber, 1997). In addition, surveillance mechanisms that identify and reduce the half-lives of aberrant mRNAs that contain nonsense codon mutations have been described (Maquat, 1995; Jacobson and Peltz, 1996). Therefore, regulation of the half-life of mRNAs can have dramatic consequences on cellular responses and functional outcomes during growth and development.

Through the application of genetics, the mechanisms and factors involved in the turnover of mRNA in *Saccharomyces cerevisiae* are beginning to be identified. Multiple pathways of mRNA turnover are present in yeast, allowing for numerous levels of regulation and fine-tuning of gene expression. One general pathway of mRNA decay involves poly(A) tail shortening followed by decapping and 5'-to-3' exonucleolytic decay (Muhlrad et al., 1994). A second general pathway involves deadenylation followed by 3'-to-5' turnover of the body

of the mRNA (Anderson and Parker, 1998). Endonucleolytic cleavage of some mRNAs has also been demonstrated (Presutti et al., 1995). Finally, another alternative decay pathway that bypasses deadenylation is involved in the translation-dependent degradation of nonsense codon-containing mRNAs (Weng et al., 1997). Several degradation enzymes and regulatory proteins that play a role in mRNA stability in yeast have been identified (Caponigro and Parker, 1996; Weng et al., 1997). Functionally significant interactions between the cap structure and the 3' poly(A) tail of yeast mRNAs have also been described (Tarun and Sachs, 1997). Whether these observations are generally applicable to mammalian cells, however, remains to be established.

In vivo observations are beginning to allow some generalizations concerning major pathways of mRNA turnover in mammalian cells. A poly(A) tail of approximately 200 bases is added to most mRNAs during processing in the nucleus (Colgan and Manley, 1997). The poly(A) tail serves at least two known functions in mRNA stability. First, in association with poly(A) binding proteins (Bernstein et al., 1989; Ford et al., 1997), it protects the mRNA from 3'-to-5' exonucleases. Second, the poly(A) tail serves as an initiation site for the turnover of the mRNA. The poly(A) tail can be progressively shortened throughout the lifetime of a mRNA in the cytoplasm. Controlling the rate of deadenylation appears to be an important regulatory point in mRNA stability (Wilson and Treisman, 1988; Xu et al., 1997). Once the poly(A) tail is shortened to approximately 30-65 bases, the body of the mRNA appears to be degraded in a rapid fashion *in vivo* without the accumulation of discernible intermediates (Chen et al., 1995; Xu et al., 1997). Little is known, however, concerning the enzymes and regulatory components involved in mammalian mRNA turnover.

In addition to the poly(A) tail, several *cis*-acting elements have been shown to play a role in mRNA stability. The 5' terminal cap structure protects the transcript from exonucleases (Furuichi et al., 1977). Several destabilizing elements (Caput et al., 1986; Shyu et al., 1989; Bonnieu et al., 1990; Peng et al., 1996), as well as stabilizing elements (Stefonovic et al., 1997), located in the body of the mRNA have also been identified. One well-characterized element that regulates mRNA stability is an AU-rich sequence (ARE) found in the 3' untranslated region of many short-lived mRNAs (Shaw and Kamen, 1986). These AREs primarily consist of AUUUA repeats or a related nonameric sequence (Lagnado et al., 1994; Zubiaga et al., 1995; Xu et al., 1997) and have been divided into three classes based on sequence characteristics and degradation kinetics (Xu et al., 1997). In general, AREs have been shown to increase the rate of deadenylation and RNA turnover in a translation-independent fashion (Chen et al., 1995; Fan et al., 1997). The underlying mechanism behind ARE function, however, remains to be determined.

Numerous proteins have been described that can bind *in vitro* to AU-rich elements (e.g. Malter, 1989; Vakalopoulou et al., 1991; Bohjanen et al., 1991; Brewer, 1991; Levine et al., 1993; Hamilton et al., 1993; Katz et al., 1994; Nakagawa et al., 1995; Ma et al., 1996), but the exact role of each factor in the process of mRNA turnover remains to be defined. The ELAV family of ARE-binding proteins is evolutionarily conserved and differentially expressed in tissues throughout the development of vertebrates (reviewed in Antic and Keene, 1997). Although ELAV proteins have been found in both the cytoplasm and the nucleus (Gao and Keene, 1996), the most ubiquitously expressed form, HuR, can shuttle between the nucleus and the cytoplasm (Fan and Steitz, 1998; Peng et al., 1998; Atasoy et al, 1998). ELAV proteins play an important role in growth and development, as the Drosophila homolog is genetically essential for development and maintenance of the nervous system

(Campos et al., 1985; Robinow and White, 1988). In addition, mammalian ELAV proteins are induced during differentiation and are distributed in RNP granules along dendrites (Gao and Keene, 1996). Several lines of evidence suggest that ELAV proteins control aspects of post-transcriptional gene expression (Gao and Keene, 1996; Koushika et al., 1996; Myer et al., 1997; Ma et al., 1997; Antic and Keene, 1998). Over-expression of ELAV family members, for example, has been shown to affect accumulation of selected mRNAs (Jain et al., 1997; Levy et al., 1998; Fan and Steitz, 1998; Peng et al., 1998). The precise role of ELAV proteins and other ARE-binding factors, however, remains to be established.

Mechanistic questions in mammalian cells are usually best approached using biochemical systems due to the inherent difficulties with mammalian cells as a genetic system. It has been difficult, however, to establish a versatile *in vitro* system to study mRNA stability and turnover. Based on *in vivo* observations and practical considerations, an optimal *in vitro* system to study the process of mRNA stability should have the following properties: First, the system should be efficient and highly reproducible. Second, minimal amounts (preferably undetectable) of RNA degradation in the system should be due to random degradation by non-specific contaminating ribonucleases. Third, deadenylation should occur before general degradation of the mRNA body is observed. Fourth, degradation of the mRNA body should occur in an apparently highly processive fashion without detectable intermediates. Fifth, regulation of the rate of overall deadenylation and degradation should be observed in a sequence-specific manner. Finally, the system should work on exogenous RNAs to allow ease of experimental manipulation.

Reported herein is the discovery of a new and useful *in vitro* mRNA stability system using cytoplasmic S100 extracts that fulfills all of the criteria listed above and possesses all of the

properties known to be involved in ARE-mediated mRNA turnover. This system has been successfully used to demonstrate a role for the AU-rich element binding proteins of the ELAV family in mRNA stability. These findings indicate that ELAV proteins can affect a default pathway of ARE-mediated degradation by either protecting the mRNA from nuclease attack or by displacing factors that otherwise mark these short-lived transcripts for degradation. This *in vitro* system allows the identification of cellular factors involved in mRNA turnover and help elucidate mechanisms involved in the post-transcriptional regulation of gene expression.

Moreover, the *in vitro* system of the invention has ready applications in high throughput assays to screen libraries of compounds to elucidate which compounds may have applications as pharmaceuticals which can modulate the stability and turnover of RNA transcripts *in vivo*, and thus be used to treat a wide variety of disease or disorders.

i. <u>Development of an *In vitro* System that Deadenylates and Degrades RNA Substrates</u>

The development of an *in vitro* system to study mRNA turnover requires the generation of a convenient source of poly(A)$^+$ RNA substrate and an active cellular extract. In order to obtain substrate RNAs that were both polyadenylated and easy to identify using standard acrylamide gel technology, a novel and versatile ligation-PCR approach that can attach a template encoding a 60 base poly(A) tail to the 3' end of DNA fragments that contain a Hind III site was used, and is described *infra*. In initial studies to develop an *in vitro* RNA stability system, a 60 base poly(A) tail was attached to a 54 base polylinker-derived sequence (Gem-A60). The small size of this polyadenylated transcript made it easy to analyze intermediates in the pathway of RNA turnover on acrylamide gels. Cellular extracts were prepared following a standard cytoplasmic S100 protocol (Dignam et al., 1983) using

hypotonically lysed Hela spinner cells with minor variations as described in the Materials and Methods.

Gem-A60 RNA was incubated in S100 extracts in the presence of ATP. As seen in Fig. 1A (left panel), very little turnover of the Gem-A60 RNA was noted after 60 minutes of incubation. This reproducible slow rate of turnover prompted us to hypothesize that an inhibitor of the deadenylation/degradation process might be present in S100 extracts. This hypothesis was based on several observations. First, previous work with nuclear extracts determined that poly(A) binding proteins were strong inhibitors of a 3'-to-5' exonuclease activity (Ford et al., 1997). Second, the activity of a partially purified mammalian deadenylase preparation was inhibited by high amounts of PABP (Korner and Wahle, 1997). Third, over-expression of PABP in *Xenopus* oocytes inhibits maturation-specific deadenylation (Wormington et al., 1996). In order to test whether excess amounts of poly(A) binding proteins were responsible for inhibiting the deadenylation of Gem-A60 RNA in S100 extracts, increasing amounts of cold poly(A) competitor RNA were added to the reaction mixtures to sequester poly(A) binding proteins. As shown in Fig 1A (right side), the addition of poly(A) competitor activated a degradation activity in the S100 extracts. The Gem-A60 RNA was shortened to a species slightly larger than the size of a deadenylated marker (Gem-A0) and approximately 30% of the input RNA was degraded. Titration experiments performed in coordination with UV cross-linking studies demonstrated that the amount of poly(A) competitor RNA required to activate the S100 extract precisely corresponded with the ability of the competitor to inhibit binding of proteins to the poly(A) tail of the substrate RNA (data not shown). Furthermore, the nucleolytic activities activated by the addition of cold poly(A) RNA as competitor to the S100 extracts were still observable at concentrations

of poly(A) >500 ng. These data suggest that the activated nuclease(s) is highly refractory to competition by poly(A).

The progressive shortening of the Gem-A60 RNA substrate observed upon incubation in S100 extract supplemented with poly(A) competitor RNA was determined to be due to a 3'-to-5', poly(A) tail-specific exonuclease based on the following observations: First, RNA substrates ^{32}P-labeled exclusively at their 5' cap structures were progressively shortened in the system in a similar fashion as uniformly labeled transcripts (compare Figs. 1A and 1B). These data suggest that the shortening of the input RNA occurred in a 3'-to-5' direction. This conclusion was confirmed by separately analyzing the 5' and 3' portions of RNA products from the *in vitro* system by RNAse H digestion prior to gel electrophoresis. As shown in Fig. 1C, the 3' portion of the substrate RNA (which consists primarily of the 60 base poly(A) tail) was clearly being degraded before any turnover of the 5' portion of the transcript was detected. After 9 minutes of incubation, 72% of the 3' fragment containing the poly(A) tail is degraded, while only 19% of the 5' fragment has been turned over. Finally, in order to ascertain whether this 3'-to-5' exonuclease activity was indeed a poly(A)-specific deadenylase, we added 15 bases of non-adenylate sequence onto the 3' end of the Gem-A60 RNA (Gem-A60-15). As seen in Fig. 1D, while the Gem-A60 transcript (which contains a 3' poly(A) tail) is an excellent substrate for the 3' exonuclease activity, the Gem-A60-15 RNA, which has its poly(A) tract internalized 15 bases, was not.

From these data it has been concluded that the addition of poly(A) competitor RNA to an S100 extract activates a deadenylase which is active on exogenous, poly(A)+ substrate RNAs. The *in vitro* system reproduces several aspects of mRNA stability observed *in vivo*. The surprising observation that the deadenylase itself is not apparently inhibited by cold

poly(A) suggests that the native enzyme may not have high affinity for its substrate. The deadenylase activity may contain additional RNA binding activities that anchor it to mRNAs, perhaps as part of a multi-component complex.

ii. <u>RNA turnover in the *in vitro* system is regulated by AU-rich instability elements.</u> It was determined whether the RNA turnover activities exhibited by the S100 extract system could be influenced or modulated by sequences in the body of the transcript in a specific manner. The relative stability of small polyadenylated RNAs containing either a 54 base polylinker sequence (Gem-A60), a 34 base AU-rich element (ARE) from TNF-α mRNA (ARE-A60), or a 72 base ARE from the c-fos mRNA (Fos-A60) was determined in the *in vitro* stability system. As shown in Figs. 2A and 2B, the turnover of both of the ARE-containing RNAs was dramatically increased compared to the Gem-A60 control transcript. To directly assess whether regulation by AREs was occurring in a sequence-specific fashion, the TNF-α-ARE was extensively mutated as described in Materials and Methods. Similar mutations in AU-rich instability elements were shown previously to greatly increase mRNA half-life *in vivo* (Myer et al., 1997). As seen in Fig 2C, mutations in the ARE reduced the rate and extent of deadenylation /degradation over 3-fold in the *in vitro* system. Thus, RNA turnover in the *in vitro* system can be regulated or modulated by AU-rich instability elements in a sequence-specific fashion.

All of the RNA substrates we have examined above contain a body of approximately 50-70 bases attached to a poly(A) tail. It was then determined whether regulated turnover using larger polyadenylated RNA substrates could be detected in the system of the invention. As shown in Fig. 2D, a polyadenylated 250 base RNA derived from the 3' UTR of the SV40 late mRNA (SV-A60) was deadenylated but inefficiently degraded in the *in vitro* system. Adding

the TNF-α-ARE to the 3' portion of this RNA (SVARE-A60) resulted in an approximate 3.5 fold increase in the rate of turnover. Finally, a nearly full length (~950 base) version of the human GM-CSF mRNA was prepared, as well as one in which the ARE was deleted (GM-CSF(-ARE)). The 3' ends of these transcripts were polyadenylated using yeast poly(A) polymerase (Martin and Keller, 1998). Gel purified RNAs were incubated in the *in vitro* stability system and aliquots were removed at the times indicated. As seen in Fig 2E, the version of the GM-CSF mRNA that contains an ARE was approximately 2.5 fold less stable than GM-CSF(-ARE) in the *in vitro* system. As seen above with other transcripts, the GM-CSF transcripts were also deadenylated in the system. Deadenylation was not observable in Fig 2E due to the lack of resolution of the gel system employed, but can be observed using formaldehyde-agarose gels (data not shown).

iii. <u>Degradation, but not deadenylation, requires ATP</u>

Transcripts with 60 adenylates at the 3' end were observed to undergo both deadenylation and turnover in the *in vitro* system. This is consistent with *in vivo* observations that suggest the poly(A) tail is shortened to about 30-65 bases before mRNA turnover is observed (Xu et al., 1997). Since degradation appeared to begin before the input transcript was completely deadenylated (eg. Fig. 2), it was difficult to quantitatively assess the effects of AU-rich elements on relative deadenylation rates. In order to try uncoupling these processes and accurately evaluate the effect of AREs on deadenylation rates in the *in vitro* system, we surveyed the cofactor requirements that might be unique to either deadenylation or turnover. Both processes were inhibited by the addition of EDTA (data not shown), suggesting a role for divalent cations. Curiously, deadenylation could occur without the addition of ATP/phosphocreatine to the system (Fig. 3A). Degradation, on the other hand, required ATP/phosphocreatine as indicated by the accumulation of deadenylated intermediates in its absence (Fig. 3A, lanes -ATP). By omitting ATP from the reaction, therefore, we were able to evaluate relative deadenylation rates in the presence or absence of an AU-rich instability element. RNAs with physiological length poly(A) tails (150-200 bases) which lack (SV-A150-200) or contain (SVARE-A150-200) an ARE were incubated in the *in vitro*

system and aliquots were analyzed at the times indicated. As seen in Fig. 3B, RNA substrates containing an ARE were deadenylated at an approximately two fold faster rate than RNAs that do not contain the instability element.

In summary, an *in vitro* mRNA stability system has been discovered that acts on exogenous substrates and faithfully reproduces all of the known *in vivo* aspects of turnover. RNAs are first deadenylated prior to degradation of the body of the transcript. Degradation of the body of the mRNA then occurs in an apparently highly processive fashion with no discernible intermediates. Deadenylation and decay rates are increased several fold by the inclusion of an AU-rich instability element. ARE regulation of RNA stability is sequence-specific and highly reproducible, as all three of the AREs we have tested in the *in vitro* system function in a similar fashion. This system should provide a valuable means to elucidate mechanistic aspects of regulated and general mRNA turnover pathways.

iv. The role of ARE binding proteins in the *in vitro* system.

The *in vitro* system described here allows evaluation of the role of ARE-binding proteins in the process of RNA deadenylation/degradation. Several proteins were found to be associated with ARE-containing RNAs in our extracts. As seen in Fig. 4A, a protein of ~30 kDa and a group of ~40 kDa proteins were specifically UV cross-linked to the short ARE-A60 transcript. A species of approximately 70 kDa was also detected when this ARE was inserted into a larger transcript (SVARE-A60; see Fig. 5B). It is possible that this 70 kDa protein was not detected on the ARE-A60 RNA because of the relatively small size of the transcript. Efforts to determine the identity of these cross linked species using available antibodies to known ARE-binding proteins revealed the presence of an ELAV protein. As shown in Fig. 4B, immunoprecipitation assays identified the 30 kDa protein as HuR (a.k.a. HuA), a member of the ELAV protein family that is ubiquitously expressed in all tissues (Good, 1995; Ma et al., 1996; Myer et al., 1997). Antisera against another RNA-binding protein of approximately 30 kDa, hnRNP A1, failed to detect any cross linked protein in our system (Fig. 4B). Two additional antisera were tested in order to identify the 40 kDa band. Antibodies to hnRNP C protein failed to detect any cross linked protein , while antisera to AUF-1 (a.k.a. hnRNP D)(Brewer, 1991) did precipitate a small amount of cross linked 40 kDa protein (data not shown). However, this cross linked product was not competed by increasing amounts of a 34 base synthetic ARE competitor RNA (data not shown). The

significance of this low level of non-specific AUF-1 cross linking in the system is unclear. It was concluded that the 30 kDa species that specifically cross links to the ARE element is HuR, a protein that has been previously suggested to play a role in ARE-mediated mRNA decay (Vakaloloupou et al, 1991; Antic and Keene, 1997; Myer et al., 1997).

Next, it was determined whether the interaction of the cross linked ARE binding proteins with the element was required to mediate instability. Synthetic ribonucleotides containing either a 34 base TNF-α ARE or randomly chosen, non-ARE sequences were used. Synthetic competitor RNAs were added in increasing amounts to the *in vitro* stability system and their effect on RNA turnover was assessed. As seen in Fig. 5A, the ARE competitor RNA completely inhibited deadenylation and degradation at 40 pm, while the non-specific RNA had no effect at similar concentrations. The ARE competitor RNA had a similar effect on the deadenylation/degradation of RNAs whether or not they contained an ARE. Thus, factors capable of interacting with AREs are important for deadenylation, and may be a part of a multi-protein deadenylase/degradation complex.

The ability of the synthetic ARE competitor RNA to block deadenylation was compared with the ability of the RNA to compete for interaction of ARE-binding proteins with the substrate transcript. EDTA was added to cross-linking assays to inhibit RNA turnover and to evaluate the effect of various levels of competitor on cross-linking/label transfer efficiency. As shown in Fig. 5B, all ARE-binding proteins (including HuR protein that could be immunoprecipitated using specific antisera prior to gel electrophoresis as shown in panel C) were specifically competed from the SV-ARE-A60 RNA substrates upon addition of 5 pm of the synthetic RNA competitor. As shown in Fig. 5A, however, 5 pm of synthetic ARE competitor RNA failed to have an appreciable effect on the rate of RNA deadenylation/degradation in the system. Hence, none of the ARE-binding proteins that could be detected by cross-linking appear to be required for deadenylation/degradation in the *in vitro* system.

v. <u>ELAV proteins prevent degradation of deadenylated transcripts in the *in vitro* system</u>

Since the ARE binding proteins we detected by cross-linking do not appear to be required for deadenylation/degradation, they may play a role in transcript stability. Consistent with this

model, recent *in vivo* data suggest that overexpression of Hel-N1 and HuR proteins can stabilize ARE-containing transcripts (Jain et al., 1997; Fan and Steitz, 1998; Peng et al., 1998). A mouse recombinant HuR protein, as well as other members of the ELAV family (Hel-N1 and Hel-N2 [a.k.a. HuB]) were produced as GST fusion proteins and added these to the *in vitro* stability system at a 10:1 molar ratio to substrate RNA. Similar data were obtained using any of the three recombinant ELAV family proteins, and only data with rHel-N1 is shown. As seen in Fig. 6A, rHel-N1 protein failed to affect deadenylation of the SVARE-A60 RNA substrate in the *in vitro* system, but stabilized a deadenylated intermediate. GST alone, or another GST-fusion protein that binds RNA (hnRNP H') had no effect on transcript stability in the *in vitro* system (Fig. 6B). As a result, it was concluded that the ELAV family of RNA binding proteins function to protect deadenylated transcripts from the degradation enzymes.

Next, it was tested whether the RNA substrate must contain an ARE in order for rELAV proteins to stabilize a deadenylated intermediate in the *in vitro* system. ARE-A60 RNA, or an unrelated but similarly sized and polyadenylated transcript, CX-A60, were incubated in the *in vitro* system in the presence or absence of rELAV proteins. As seen in Fig. 6C, rHelN1 (or other rELAV proteins [data not shown]) stabilized the deadenylated intermediate only from RNAs that contain an ARE binding site. Thus, the stabilization of deadenylated intermediates by ELAV proteins requires an ARE. Furthermore, ELAV proteins can stabilize a deadenylated intermediate whether the ARE is located at the 3', 5' or central positions of the 250 base SVARE-A60 RNA. These data indicate that the ARE-ELAV protein complex probably is not simply preventing turnover through steric blocking of an end of the transcript, thereby preventing exonuclease access.

Set forth herein is a novel and useful *in vitro* RNA stability system that faithfully reproduces many known aspects of *in vivo* mRNA turnover in mammalian cells. Exogenous RNA substrates are deadenylated before degradation of the RNA body occurs in an apparently highly processive fashion without detectable intermediates. Furthermore, the rates of RNA deadenylation and degradation are regulated by AU-rich elements in the system in a sequence-specific manner. The system of the invention has been successfully used to determine a role for the ELAV family of ARE binding proteins in the stability of

deadenylated transcripts by specifically blocking the degradation step. These data illustrate the value of the system to address the mechanism of regulated mRNA turnover.

The *in vitro* system described in this report has several key technical advantages that significantly increase its utility. First, the system is highly reproducible and uses standard S100 cytoplasmic extracts from Hela spinner cells. In fact, nine independent preparations of S100 extract that all function in the assay in a similar fashion have been tested. The only difference among extracts appears to be in the kinetics of turnover (e.g. compare the slight differences in the pattern of turnover of Gem-A60 RNA in Fig. 1A with the pattern observed in Fig. 1D). Second, the extracts exhibit minimal background degradation of RNA due to non-specific nucleases. This lack of noise in the system significantly contributes to its reproducibility. Another key element of the system is that is uses exogenous polyadenylated RNAs as substrates. This property affords variety in RNA substrate preparation and sequence manipulation. Fourth, the system exhibits sequence-specific regulation by AU-rich elements in the absence of translation. In total, these technical advantages make the system a valuable reagent to identify components involved in mRNA turnover and address the mechanism of regulated mRNA stability.

The addition of poly(A) competitor RNA was required to activate S100 extracts to efficiently deadenylate and degrade RNAs in a regulated manner. Titration of cold poly(A) demonstrated that the extracts became activated for deadenylation/degradation when sufficient competitor was added to substantially reduce cross linking of a 70 kDa poly(A) binding protein to the poly(A) tail of the radiolabeled substrate RNA (data not shown).

Surprisingly, the deadenylation in the extracts remain active even in the presence of >500 ng of poly(A). Commercial poly(A) preparations prepared with polynucleotide phosphorylase, therefore, do not appear to be able to interact with and sequester the deadenylase enzyme. These data suggest that the deadenylase activity is either in extraordinary concentrations in the extracts or may not have a strong affinity for its substrate. In conjunction with this, it has been observed that an increase in deadenylation rate of ARE containing RNAs (Figs. 2 and 3), as well as the ability of the ARE competitor RNA to inhibit deadenylation of non-ARE containing substrates. These data suggest that ARE-binding proteins may be associated with the deadenylase activity.

Moreover, HuR protein, a ubiquitously expressed member of the ELAV family of RNA binding proteins (Good, 1995; Ma et al., 1996; Myer et al., 1997; Antic and Keene, 1997), has been identified as one of the major ARE binding factors in the system of the invention. Also, the system of the invention has been successfully used to detect weak binding to AUF-1 (hnRNP D), a protein previously speculated to be involved in regulated mRNA decay *in vitro* (DeMaria and Brewer, 1996). AUF-1, therefore, does not appear to play a significant role in transcript instability in our system. ELAV proteins are not required for deadenylation/degradation, but rather play a role in the stability of deadenylated RNAs that contain an ARE (Fig. 6). These data suggest that in addition to its effect on deadenylation rates (Chen et al., 1995; Xu et al., 1997), the ARE influences the efficiency of turnover of the body of the mRNA. *In vivo* observations (Chen et al., 1995; Xu et al., 1997; Peng et al., 1998) also support the conclusion that ARE influences mRNA degradation rates.

ELAV proteins, therefore, appear to regulate mRNA stability *in vitro*, an observation consistent with *in vivo* transfection studies. The ELAV family comprises four members, three of which are expressed in a tissue or developmental specific manner (reviewed in Antic and Keene, 1997). Tissue-specific ELAV proteins are also localized primarily to the cytoplasm, while the ubiquitous HuR protein is predominantly nuclear and can redistribute to the cytoplasm (Atasoy et al., 1998; Peng et al., 1998; Fan and Steitz, 1998). It has been suggested that differentially expressed ELAV proteins play a role in regulating the stability of both nuclear and cytoplasmic RNA, thereby fine tuning gene expression in specific developmental states (Gao and Keene, 1996; Antic and Keene, 1998).

The competition data shown in Fig. 5 clearly demonstrate that factors associated with the ARE are required for deadenylation/degradation of substrate RNAs. Based on the kinetics of competition, these factors must either be much more abundant than the cross-linkable ARE binding proteins like HuR, or interact with the ARE with a much lower affinity. We favor the latter model, and suggest that these factors are part of a multi-component complex that includes the deadenylase and degradation enzymes. Through multiple cooperative interactions, these weak ARE binding components may allow efficient assembly of the deadenylase/degradation complex on ARE containing transcripts while still allowing the complex to assemble, albeit less effectively, on non-ARE containing RNAs. The RNA

binding components of this proposed complex also may have affinity for other non-ARE instability elements (e.g. Peng et al., 1996).

The observation that endogenous HuR protein in S100 extracts set forth herein can be cross-linked to ARE-containing RNA substrates (Fig. 5) makes it surprising that an ARE can function as a destablizing element in the *in vitro* assay. Since HuR protein is predominantly nuclear, however, only low levels of the protein are likely to be present in our cytoplasmic extracts. This low level of HuR protein is probably unable to efficiently compete with destablizing factors for binding to the ARE. In fact, sequestration of the HuR protein by the addition of low levels of synthetic ARE competitor RNA does lead to an increased rate of turnover of ARE-containing RNAs in the *in vitro* system. As shown in Fig. 5A, the amount of SVARE-A60 RNA remaining after 30 min. in the system in the absence of competitor RNA (lane 0) is approximately 20% greater than when the assay is done in the presence of 5 pm of ARE competitor RNA (lane 5 pm). The removal or sequestration of HuR protein in S100 extracts, therefore, may be necessary in order to observe regulated deadenylation and degradation in some instances.

Materials and Methods

Transcription templates and RNAs

RNAs were produced by *in vitro* transcription using SP6 polymerase (Melton et al., 1984) in the presence of ^{7m}GpppG cap analog and radiolabeled UTP or ATP as indicated. All transcripts were gel purified prior to use. For RNAs labeled exclusively at the 5' cap, transcription reactions were performed in the absence of cap analog and radioactive nucleotides. Capping was then performed using guanyltransferase (BRL) and radiolabled GTP according to the manufacturer's recommendations. The sequence of short RNAs used as substrates in the *in vitro* system is shown in Table 1.

Transcription templates were derived as follows (Please note that all synthetic oligonucleotides used as transcription templates shown below contain a 24 base SP6 promoter fragment at their 5' ends): Gem-A0 RNA was produced from Hind III cut pGem4 (Promega). Gem-A60-15 RNA was produced from the PCR product used to produce Gem-A60 RNA (see below) without removing the primer binding site with Ssp I. Templates

for ARE-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5'-ATTTAGGTGACACTATAGAATACACATTATTTATTATTTATTTATTATTTATTTA TTTA-3' (SEQ ID NO:1) and its appropriate complement. Templates for MT-ARE-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5'-ATTTAGGTGACACTATAGAATACACGTTAGTATTCATTTGTTTACTATTGATTTC TTTA-3' (SEQ ID NO:2) and its appropriate complement. Templates for Fos-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5'-ATTTAGGTGACACTATAGAATACACAAATTTTATTGTGTTTTTAATTTATTTATT AAGATGGATTCTC-3' (SEQ ID NO:3) and its appropriate complement. The template for SV-A0 RNA was Hind III cut pSVL-Gem (Wilusz et al., 1988). Templates for SVARE-A0 RNA were generated by inserting the TNF-α ARE containing oligonucleotide 5'-ATTATTTATTATTTATTTATTATTTATTATTTA (SEQ ID NO:4) and its appropriate complement between the PstI and Hind III sites of pSVL-Gem (located near the 3' end of the RNA). SVARE-A0 RNA was transcribed from Hind III linearized DNA. The template for GM-CSF (+ARE) RNA was EcoRI cut pGM-CSF (Shaw and Kamen, 1986). The template for GM-CSF (-ARE) RNA was NcoI cut pGM-CSF. Templates for CX-A0 RNA were generated by hybridizing the synthetic oligonucleotide 5'-ATTTAGGTGACACTATAGAATACACCCCAACGGGCCCTCCCTCCCCTCCTTGCA CCATCATCGCATCACG (SEQ ID NO:5) and its appropriate complement.

Synthetic RNAs used in competition studies were made by the NJMS Molecular Core Facility and contained the following sequences: ARE: 5'AUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUA (SEQ ID NO:6); Non-specific competitor: 5'-GUCACGUGUCACC (SEQ ID NO:7).

Addition of Poly(A) tails to transcripts

A template for a 60 base poly(A) tail was added to DNA templates using a ligation/PCR protocol have recently been described (Ford et al., 1997). Briefly, all of the templates described above contain a Hind III site that is used to generate the 3' end of the RNA. The synthetic oligonucleotide 5'-AGCTA$_{60}$TATTGAGGTGCTCGAGGT (SEQ ID NO:8) and its appropriate complement were generated, hybridized, and ligated to Hind III cut DNA templates. Ligation products were amplified using an SP6 promoter primer (5'-CATACGATTTAGGTGACACTATAG (SEQ ID NO:9)) and a primer specific for the 3'

end of the ligated oligonucleotide (5'-ACCTCGAGCACCTC (SEQ ID NO:10)). Amplified products were purified on Centricon 100 columns, cut with SspI, and used as templates for SP6 polymerase generate RNAs carrying the 'A60' designation.

Poly(A) polymerase (Amersham) was used to add 150-200 base poly(A) tails onto transcripts. RNAs were incubated with enzyme according to the manufacturer's recommendations on ice for 5-8 min. Following the reaction, RNAs were extracted with phenol-chloroform, ethanol precipitated, and purified on 5% acrylamide gels containing 7M urea to obtain RNAs with the appropriate amount of poly(A) at the 3' end.

S100 extract production

Cytoplasmic extracts were prepared from Hela spinner cells grown in JMEM supplemented with 10% horse serum as described by Dignam et al (1983) with the following two modifications. First, following centrifugation at 100,000 x g for 1 hr, the supernatant was adjusted to 10% glycerol prior to dialysis. Second, dialysis times were shortened to 30 min. Extracts were stored at −80°C.

In vitro RNA deadenylation/degradation system

Typically, approximately 200,000 cpm (~50 fm) of gel purified RNA is used per reaction. In comparative studies, equal molar amounts of transcripts were used. A typical 14.25 μl reaction mixture contains 3.25 μl of 10% polyvinyl alcohol, 1 μl of a 12.5 mM ATP/ 250 mM phosphocreatine mixture, 1 μl of 500 ng/ul poly(A) (Pharmacia), 1 μl of RNA and 8 μl of dialyzed extract. Reactions were incubated at 30° C for the times indicated and stopped by the addition of 400 μl of stop buffer (400 mM NaCl, 25 mM Tris-Cl, pH 7.6, 0.1% SDS). Reaction mixtures were phenol extracted, ethanol precipitated and analyzed on a 5% acrylamide gel containing 7M urea. All quantitation was performed using a Molecular Dynamics Phosphorimager.

Recombinant ELAV proteins (HuR, Hel-N1 and Hel-N2) were made as GST-fusion proteins in *E. coli* and purified using glutathione-sepharose affinity chromatography according to the manufacturer's recommendations (Levine et al, 1993).

RNase H digestion

ARE-A60 RNA, radiolabeled at A residues, was incubated in the *in vitro* stability system for the times indicated. RNA products were phenol extracted and concentrated by ethanol precipitation. The sample was resuspended in a final volume of 30 µl containing 20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 picomoles of the antisense oligonucleotide 5'-AGTTAAATAAAT (SEQ ID NO:11), and 1 unit of RNase H. Reactions were incubated at 37°C for 30 min. and products were analyzed on a 5% acrylamide gel containing 7 M urea.

UV Cross linking and Immunoprecipitations

UV cross linking/label transfer experiments were performed as described previously using a Sylvania G15T8 germicidal light (Wilusz and Shenk, 1988). Cross linking experiments were done in the presence of 25 mM EDTA to inhibit RNA turnover to allow for accurate comparisons between samples. Following digestion with RNAses A, T1 and T2, cross linked proteins were analyzed on 10% acrylamide gels containing SDS.

For immunoprecipitation analysis following UV cross linking and RNAse treatment, 300 µl of RIPA buffer (0.15M NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris-Cl, pH 7.6) was added to samples. Following a brief centrifugation in a microfuge, precleared samples were incubated on ice with antibodies for 1 hr. Antigen-antibody complexes were collected using formalin fixed, washed protein-A positive *S. aureus* cells, washed five times using RIPA buffer, and analyzed on a 10% acrylamide gel containing SDS. Antibodies specific for GRSF (Qian and Wilusz, 1994) and hnRNP A1 (Wilusz and Shenk, 1990) have been described previously. The preparation and characterization of rabbit polyclonal antibodies specific for HuR will be described elsewhere (Atasoy et al., 1998).

Aghib, D.F., Bishop, J.M., Ottolenghi, S., Guerrasio, A., Serra, A., and Saglio, G. 1990. A 3' truncation of myc caused by chromosomal translocation in a human T-cell leukemia increases mRNA stability. Oncogene **5:** 707-711.

Anderson, J.S.J., and Parker, R. 1998. The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SK12 DEVH box protein and 3' to 5' exonuclease of the exosome complex. EMBO J. **17:** 1497-1506.

Antic, D., and Keene, J.D. 1997. Embryonic lethal abnormal visual RNA-binding proteins involved in growth, differentiation, and posttranscriptional gene expression. Am. J. Hum. Genet. **61:** 273-278.

Antic, D., and Keene, J.D. 1998. Messenger ribonucleoprotein complexes containing human ELAV proteins: interactions with cytoskeleton and translational apparatus. J. Cell. Sci. **111:** 183-197.

Atasoy, U., Watson, J., Patel, D., and Keene, J.D. 1998. Ubiquitously-expressed ELAV RNA binding protein, HuA, is upregulated during serum stimulation and T cell activation and undergoes nuclear / cytoplasmic shuttling. J. Cell Sci., In Press.

Bernstein, P., Peltz, S.W., and Ross, J. 1989. The poly(A)-poly(A) binding protein complex is a major determinant of mRNA stability in vitro. Mol. Cell. Biol. **9:** 659-670.

Bonnieu, A., Roux, P., Marty, L., Jeanteur, P., and Piechaczyk 1990. AUUUA motifs are dispensable for rapid degradation of the mouse c-myc mRNA. Oncogene **5:** 1585-1588.

Bohjanen, P.R., Bronislawa, P., June, C.H., Thompson, C.B., and Lindsten, T. 1991. An inducible cytoplasmic factor (AU-B) binds selectively to AUUUA multimers in the 3' untranslated region of lymphokine mRNA. Mol. Cell. Biol. **11:** 3288-3295.

Brewer, G. 1991. An A+U rich element RNA-binding factor regulates c-myc mRNA stability in vitro. Mol. Cell. Biol. **11:** 2460-2466.

Brown, C.E., Tarun Jr., S.Z., Boeck, R., and Sachs, A.B. 1996. PAN3 encodes a subunit of the Pab1p-dependent poly(A) nuclease in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:** 5744-5753.

Campos, A.R., Grossman, D., and White, K. 1985. Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects: a developmental-genetic analysis. J. Neurogenet. **2:**197-218.

Caponigro, G. and Parker, R. 1996. Mechanism and control of mRNA turnover in Saccharomyces cerevisiae. Microbiol. Rev. **60:** 233-249.

Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. 1986. Identification of a common nucleotide sequence in the 3'-untranslated regions of mRNA molecules specifying inflammatory mediators. Proc. Natl. Acad. Sci. USA **83:** 1670-1674.

Chen, C.-Y.A, Xu, N., and Shyu, A.-B. 1995. mRNA decay mediated by two distinct AU-rich elements from c-fos and GM-CSF transcripts: different deadenylation kinetics and uncoupling from translation. Mol. Cell. Biol. **15:** 5777-5788.

Colgan, D.F., and Manley, J.L. 1997. Mechanism and regulation of mRNA polyadenylation. Genes Dev. **11:** 2755-2766.

DeMaria, C.T., and Brewer, G. 1996. AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. J. Biol. Chem. **271:** 12179-12184.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl. Acids Res. **11:** 1475-1489.

Fan, X.C., Myer, V.E., and Steitz, J.A. 1997. AU-rich elements target small nuclear RNAs as well as mRNAs for rapid degradation. Genes Dev. **11:** 2557-2568.

Fan, X.C., and Steitz, J.A. 1998. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. EMBO J. **17:** 3448-3460.

Ford, L.P., Bagga, P.S., and Wilusz, J. 1997. The poly(A) tail inhibits the assembly of a 3'-to-5' exonuclease in an in vitro RNA stability system. Mol. Cell. Biol. **17:** 398-406.

Furuichi,Y., LaFiandra, A., and Shatkin, A.J. 1977. 5'-terminal structure and mRNA stability. Nature **266:** 235-239.

Gao, F.B., and Keene, J.D. 1996. Hel-N1/Hel-N2 proteins are bound to poly(A)+ mRNA in granular RNP structures and are implicated in neuronal differentiation. J. Cell Sci. **109:** 579-589.

Good, P.J. 1995. A conserved family of elav-like genes in vertebrates. Proc. Natl. Acad. Sci. USA. **92:** 4557-4561.

Hamilton, B.J., Nagy, E., Malter, J.S., Arrick, B.A., and Rigby, W.F.C. 1993. Association of heterogeneous nuclear ribonucleoprotein A1 and C proteins with reiterated AUUUA sequences. J. Biol. Chem. **268:** 8881-8887.

Holcik, M., and Liebhaber, S.A. 1997. Four highly stable eukaryotic mRNAs assemble 3' untranslated region RNA-protein complexes sharing cis and trans components. Proc. Natl. Acad. Sci. USA **94:** 2410-2414.

Jain, R.G., Andrews, L.G., McGowan, K.M., Pekala, P.H., and Keene, J.D. 1997. Ectopic expression of Hel-N1, an RNA binding protein, increases glucose transporter (GLUT 1) expression in 3T3-L1 adipocytes. Mol. Cell. Biol. **17:** 954-962.

Jacobson, A., and Peltz, S.W. 1996. Interrelationships between the pathways of mRNA decay and translation in eukaryotic cells. Annu. Rev. Biochem. **65:** 693-740.

Katz, D.A., Theodorakis, N.G., Cleveland, D.W., Lindsten, T., and Thompson, C.B. 1994. AU-A, an RNA binding activity distinct from hnRNP A1, is selective for AUUUA repeats and shuttles between the nucleus and the cytoplasm. Nucl. Acids Res. **22:** 238-246.

Korner, C.G., and Wahle, E. 1997. Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. J. Biol. Chem. **272:** 10448-10456.

Koushika, S.P., Lisbon, M.J., and White, K. 1996. ELAV, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. Curr. Biol. **6:** 1634-1641.

Lagnado, C.A., Brown, C.Y., and Goodall, G.J. 1994. AUUUA is not sufficient to promote poly(A) shortening and degradation of a mRNA: the functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). Mol. Cell. Biol. **14:** 7984-7995.

Levine, T.D., Gao, F., King, P.H., Andrews, L.G., and Keene, J.D. 1993. Hel-N1: an autoimmune RNA-binding protein with specificity for 3' uridylate-rich untranslated regions of growth factor mRNAs. Mol. Cell. Biol. **13:** 3494-3504.

Levy, N.S., Chung, S., Furneaux, H., and Levy, A.P. 1998. Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J. Biol. Chem. **273:** 6417-6423.

Ma, W.-J., Cheng, S., Wright, A., Campbell, C., and Furneaux, H. 1996. Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. J. Biol. Chem. **271:** 8144-8151.

Ma, W.-J., Cjung, S., and Furneaux, H. 1997. The elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA. Nucl. Acids Res. **25:** 3564-3569.

Malter, J.S. 1989. Identification of an AUUUA-specific messenger RNA binding protein. Science **246:** 664-666.

Martin, G., and Keller, W. 1998. Tailing and 3'-end labeling of RNA with yeast poly(A) polymerase and various nucleotides. RNA **4:** 226-230.

Maquat, L.E. 1995. When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. RNA **1:** 453-465.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids Res. **12:** 7035-7056.

Muhlrad, D., Decker, C.J., and Parker, R. 1994. Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5' to 3' digestion of the transcript. Genes Dev. **8:** 855-866.

Myer, V.E., Fan, X.C., and Steitz, J.A. 1997. Identification of HuR as a protein implicated in AUUUA-mediated mRNA decay. EMBO J. **16:** 2130-2139.

Nakagawa, J., Waldner, H., Meyer-Monard, S., Hofsteenge, J., Jeno, P., and Moronoi, C. 1995. AUH, a novel gene encoding an AU-specific RNA binding protein with intrinsic enoyl-CoA hydralase activity. Proc. Natl. Acad. Sci. USA **92:** 2051-2055.

Peng, S. S.-Y., Chen, C.-Y.A., and Shyu, A.-B. 1996. Functional characterization of a non-AUUUA AU-rich element from the c-jun proto-oncogene mRNA: evidence for a novel class of AU-rich elements. Mol. Cell. Biol. **16:** 1490-1499.

Peng, S.-Y., Chen, C.-Y.A., Xu, N., and Shyu, A.-B. 1998. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J. **17:** 3461-3470.

Presutti, C., Villa, T., Hall, D., Pertica, C., and Bozzoni, I. 1995. Identification of the cis-elements mediating the autogenous control of ribosomal protein L2 mRNA stability in yeast. EMBO J. **14:** 4022-4030.

Qian, Z., and Wilusz, J. 1994. GRSF-1: a poly(A)+ mRNA binding protein which interacts with a conserved G-rich element. Nucl. Acids Res. **22:** 2334-2343.

Ross, J. 1995. mRNA stability in mammalian cells. Microbiol. Rev. **59:** 16-95.

Robinow, S., and White, K. 1988. The locus elav of Drosophila melanogaster is expressed in neurons at all developmental stages. Dev. Biol. **126:** 294-303.

Schiavi, S.C., Belasco, J.G., and Greenberg, M.E. 1992. Regulation of proto-oncogene mRNA stability. Biochem. Biophys Acta **1114:** 95-106.

Shaw, G., and Kamen, R. 1986. A conserved AU rich sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46:** 659-667.

Shyu, A.-B., Belasco, J.G., and Greenberg, M.E. 1989. The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes Dev. **3:** 60-72.

Stefanovic, B., Hellerbrand, C., Holcik, M., Briendl, M., Liebhaber, S.A., and Brenner, D.A. 1997. Posttranscriptional regulation of collagen a1(I) mRNA in hepatic stellate cells. Mol. Cell. Biol. **17:** 5201-5209.

Tarun, S.Z., and Sachs, A.B. 1997. Association of the yeast poly(A) tail binding protein with translation initiation factor eif-4G. EMBO J. **15:** 7168-7177.

Vakalopoulou, E., Schaack, J., and Shenk, T. 1991. A 32-kilodalton protein binds to AU-rich domains in the 3' untranslated regions of rapidly degraded mRNAs. Mol. Cell. Biol. **11:** 3355-3364.

Weng, Y., Ruiz-Echevarria, M.J., Zhang, S., Cui, Y., Czaplinski, K., Dinman, J.D., and Peltz, S.W. 1997. Characterization of the nonsense-mediated mRNA decay pathway and its effect on modulating translation termination and programmed frameshifting. In "mRNA Metabolism and Post-Transcriptional Gene Regulation". J. B. Harford, D.R. Morris, eds., Wiley-Liss, Inc., New York, pp. 241-263.

Wilson, T., and Treisman, R. 1988. Removal of poly(A) tail and consequent degradation of c-fos mRNA facilitated by 3' AU-rich sequence. Nature **366:** 396-399.

Wilusz, J., and Shenk, T. 1988. A 64 kDa nuclear protein binds to RNA segments that include the AAUAAA polyadenylation motif. Cell **52:** 221-228.

Wilusz, J., Feig, D.I., and Shenk, T. 1988. The C proteins of heterogeneous nuclear ribonucleoprotein complexes interact with RNA sequences downstream of polyadenylation cleavage sites. Mol. Cell. Biol. **8:** 4477-4483.

Wilusz, J., and Shenk, T. 1990. A uridylate tract mediates efficient heterogeneous nuclear ribonucleoprotein C protein-RNA cross-linking and functionally substitutes for the downstream element of the polyadenylation signal. Mol. Cell. Biol. **10:** 6397-6407.

Wormington, M., Searfoss, A.M., and Hurney, C.A. 1996. Overexpression of poly(A) binding protein prevents maturation-specific deadenylation and translational inactivation in Xenopus oocytes. EMBO J. **15:** 900-909.

Xu, N., Chen, C.-Y.A., and Shyu, A.-B. 1997. Modulation of the fate of cytoplasmic mRNA by AU-rich elements: key sequence features controlling mRNA deadenylation and decay. Mol. Cell. Biol. **17:** 4611-4621.

Zubiaga, A M., Belasco, J.G., and Greenberg, M.E. 1995. The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. Mol. Cell. Biol. **15:** 2219-2230.

The present invention is not to be limited in scope by the specific embodiments describe herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. An *in vitro* system capable of recapitulating regulated RNA turnover of an exogenously added preselected target RNA sequence comprising a cell extract and said target RNA sequence.

2. The system of claim 1 wherein said regulated RNA turnover is selected from the group consisting of AU-rich element regulated RNA turnover and C-rich element regulated turnover.

3. The system of claim 1 wherein said cell extract is isolated from lysed eukaryotic cells or tissues.

4. The system of claim 3 wherein said cell extract is obtained from a cell line selected from the group consisting of HeLa cells and a T cell line.

5. The system of claim 1 wherein said cell extract is prepared from cells comprising foreign nucleic acid.

6. The system of claim 1 wherein said cell extract is prepared from cells which are infected, stably transfected, or transiently transfected.

7. The system of claim 1 wherein said cell extract is partially purified.

8. The system of claim 1 wherein said cell extract is depleted of activity of proteins that bind polyadenylate.

9. The system of claim 8 wherein said cell extract depleted of activity of proteins that bind polyadenylate is prepared by a method selected from the group consisting of:
   (a) addition to said system of polyadenylate competitor RNA;
   (b) sequestration of proteins that bind polyadenylate;
   (c) addition of a proteinase that inactivates a protein that bind to polyadenylate; and

(d) addition of an agent that prevents the interaction between polyadenylate and an endogenous macromolecule that binds to polyadenylate

10. The system of claim 9 wherein said sequestration of proteins that bind polyadenylate is achieved by treatment of said extract with an material that depletes macromolecules that bind polyadenylate selected from the group consisting of antibodies to proteins that bind polyadenylate, polyadenylate, and the combination thereof.

11. The system of claim 10 wherein said material is attached to a matrix.

12. The system of claim 1 wherein said target RNA sequence is selected from the group of synthetic RNA, naturally occurring RNA, messenger RNA, chemically modified RNA, and RNA-DNA derivatives.

13. The system of claim 12 wherein said target RNA sequence comprises a 5' cap and a 3' polyadenylate sequence.

14. The system of claim 1 wherein said target RNA sequence is selected from the group consisting of unlabeled target RNA sequence, labeled target RNA sequence, and the combination thereof.

15. The system of claim 14 wherein said labeled target RNA sequence is labeled with a moiety is selected from the group consisting of a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties.

16. The system of claim 1 additionally comprising exogenously added nucleotide triphosphate.

17. The system of claim 16 wherein said nucleotide triphosphate is ATP.

18. The system of claim 1 further comprising a reaction enhancer.

19. The system of claim 18 wherein said reaction enhancer is selected from the group consisting of polyvinyl alcohol, polyvinylpyrrolidone and dextran.

20. The system of claim 19 wherein said reaction enhancer is polyvinyl alcohol.

21. A method for identifying an agent capable of modulating the stability of a target RNA sequence comprising

   (A) providine the system of claim 1;
   (B) introducing said agent into said system;
   (C) determining the extent of turnover of said target RNA sequence; and
   (D) identifying an agent able to modulate the extent of said turnover as capable of modulating the stability of said target RNA sequence.

22. The method of claim 21 wherein said system additionally comprises nucleotide triphosphate.

23. The method of claim 22 wherein said nucleotide triphosphate is ATP.

24. The method of claim 21 wherein said agent is an RNA stability modifying molecule.

25. The method of claim 21 wherein said target RNA sequence is selected from the group consisting of unlabeled target RNA sequence, labeled target RNA sequence, and the combination thereof.

26. The method of claim 25 wherein said labeled RNA sequence is labeled with a moiety is selected from the group consisting of a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties.

27. The method of claim 21 wherein said monitoring the extent of turnover of said target RNA sequence comprises determining the extent of degradation of said labeled target RNA.

28. The method of claim 21 wherein said modulating the stability of a target RNA sequence increases the stability of said target RNA sequence.

29. The method of claim 21 wherein said modulating the stability of a target RNA sequence decreases the stability of said RNA sequence.

30. The method of claim 21 wherein said agent is capable of modulating the activity of a AU rich element binding protein or a C-rich element binding protein.

31. The method of claim 30 wherein said AU rich element binding protein is selected from the group consisting of a member of the ELAV protein family; AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B.

32. The method of claim 31 wherein said member of the ELAV protein family is selected from the group consisting of HuR, Hel-N1, HuC and HuD.

33. A method for identifying an agent capable of modulating the stability of a target RNA sequence in the presence of an exogenously added RNA stability modifier comprising
   (a) providing the system of claim 1;
   (b) introducing said RNA stability modifier into said system;
   (c) introducing said agent into said system;
   (d) determining the extent of turnover of said target RNA sequence; and
   (e) identifying an agent able to modulate the extent of said turnover as capable of modulating the stability of said target RNA sequence in the presence of said exogenously added RNA stability modifier.

34. The method of claim 33 wherein said system additionally comprises nucleotide triphosphate.

35. The method of claim 34 wherein said nucleotide triphosphate is ATP.

36. The method of claim 33 wherein said target RNA sequence is selected from the group consisting of unlabeled target RNA sequence, labeled target RNA sequence, and the combination thereof.

37. The method of claim 36 wherein said labeled RNA sequence is labeled with a moiety is selected from the group consisting of a fluorescent moiety, a visible moiety, a radioactive moiety, a ligand, and a combination of fluorescent and quenching moieties.

38. The method of claim 33 wherein said determining the extent of turnover of said target RNA sequence comprises determining the extent of degradation of said labeled target RNA.

39. The method of claim 33 wherein said RNA stability modifier increases the stability of said target RNA sequence.

40. The method of claim 39 wherein said agent decreases the stability of said target RNA sequence increased by said RNA stability modifier.

41. The method of claim 33 wherein said RNA stability modifier decreases the stability of said target RNA sequence.

42. The method of claim 41 wherein said agent increases the stability of said target RNA sequence decreased by said RNA stability modifier.

43. The method of claim 33 wherein said agent is capable of modulating the activity of a AU rich element binding protein or a C-rich element binding protein.

44. The method of claim 43 wherein said AU rich element binding protein is selected from the group consisting of a member of the ELAV protein family; AUF1; tristetrapolin; AUH; TIA; TIAR; glyceraldehyde-3-phosphate; hnRNP C; hnRNP A1; AU-A; and AU-B.

45. The method of claim 44 wherein said member of the ELAV protein family is selected from the group consisting of HuR, Hel-N1, HuC and HuD.

46. A method for identifying an agent capable of modulating the deadenylation of a target RNA sequence comprising

(A) providing the system of claim 1 in the absence of a nucleotide triphosphate;

(B) introducing said agent into said system;

(C) monitoring the deadenylation of said target RNA sequence in said system; and

(D) identifying an agent able to modulate the extent of said deadenylation as capable of modulating the deadenylation of said target RNA sequence.

47. A method for identifying an agent capable of modulating the deadenylation and degradation of a target RNA sequence comprising

(A) providing the system of claim 1 in the presence of a nucleotide triphosphate;

(B) introducing said agent into said system;

(C) monitoring the deadenylation and degradation of said target RNA sequence in said system; and

(D) identifying an agent able to modulate the extent of said deadenylation and degradation as capable of modulating the deadenylation and degradation of said target RNA sequence.

48. A method for identifying an agent capable of modulating cell growth or cell differentiation in a mammal comprising determining the ability of said agent to modulate the stability of a target RNA sequence involved in the modulation of cell growth or differentiation in accordance with claim 19.

49. The method of claim 48 wherein said agent capable of modulating cell growth or cell differentiation intervenes in cellular transformation.

50. The method of claim 48 wherein said agent capable of modulating cell growth or cell differentiation intervenes in immune dysregulation.

51. A method for identifying, characterizing or isolating an endogenous molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof comprising

(A) providing the system of claim 1;

(B) introducing said protein suspected of participating in the regulation of RNA turnover into said system;

(C) monitoring the stability of said target RNA sequence in said system; and

(D) identifying, characterizing or isolating said endogenous molecule able to modulate said deadenylation or degradation as capable of participating in the deadenylation or degradation of RNA or regulation thereof.

52. The method of claim 51 wherein said molecule suspected of participating in the deadenylation or degradation of RNA or regulation thereof is protein or RNA.

53. A kit for monitoring the stability of a preselected target RNA sequence under conditions capable of recapitulating regulated RNA turnover, said kit comprising:

(a) cell extract depleted of activity of proteins that bind polyadenylate;

(b) other reagents; and

(c) directions for use of said kit.

54. The kit of claim 53 further comprising nucleotide triphosphates, a reaction enhancer, a target RNA sequence, or any combination thereof.

55. A method for identifying an agent capable of modulating the degradation a target RNA sequence in the absence of deadenylation comprising

(A) providing a cell extract in the presence of a nucleotide triphosphate;

(B) introducing said agent into said cell extract; and

(C) monitoring the degradation of said target RNA sequence in said extract.

FIG. 1A

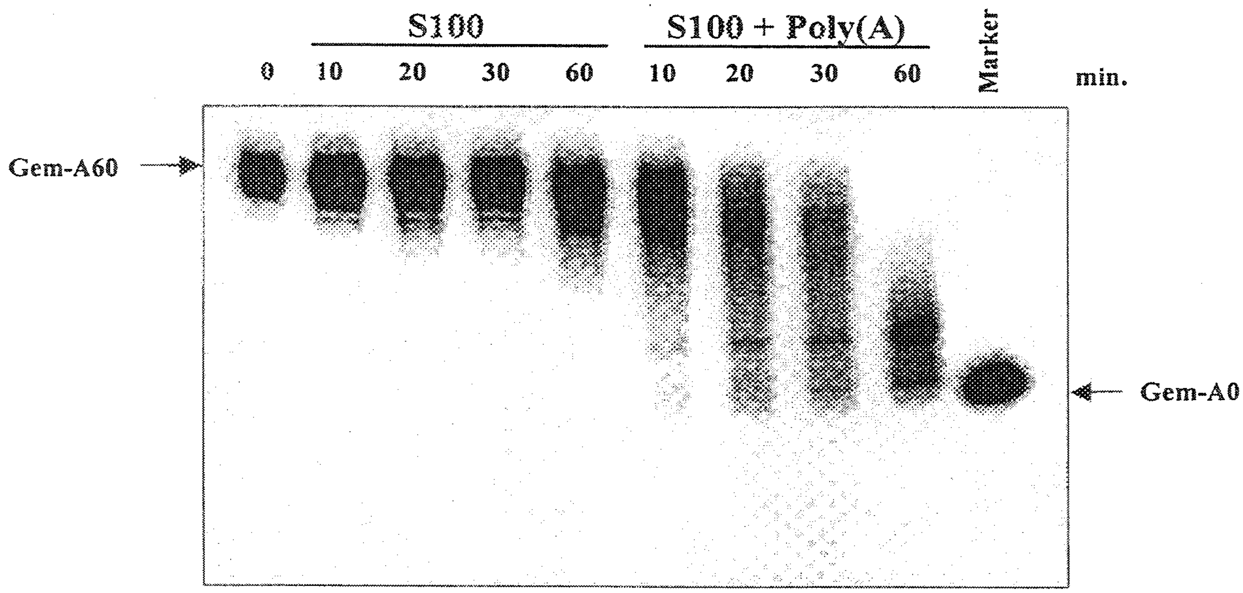

S100
S100 + Poly(A)
0
10
20
30
60
10
20
30
60
Marker
min.
Gem-A60
Gem-A0

FIG. 1B

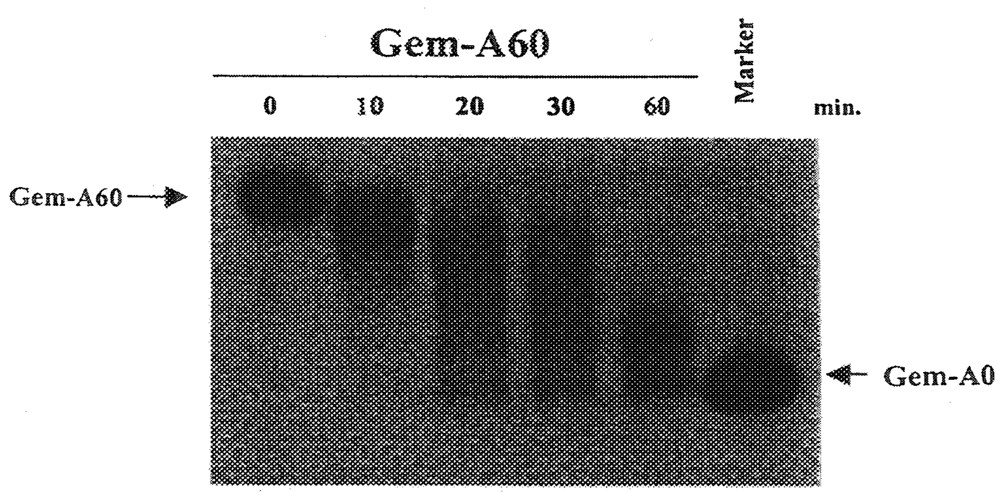

Gem-A60
0
10
20
30
60
Marker
min.
Gem-A60
Gem-A0

FIG. 1C

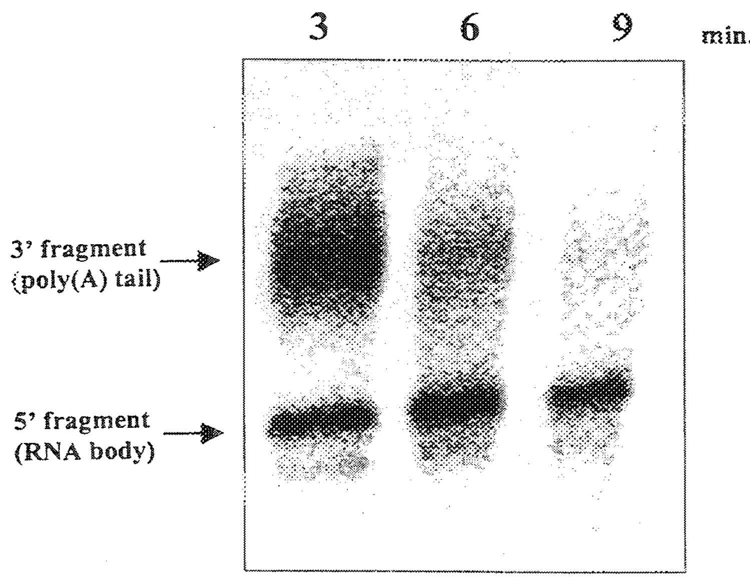

3
6
9
min.
3' fragment (poly(A) tail)
5' fragment (RNA body)

FIG. 1D

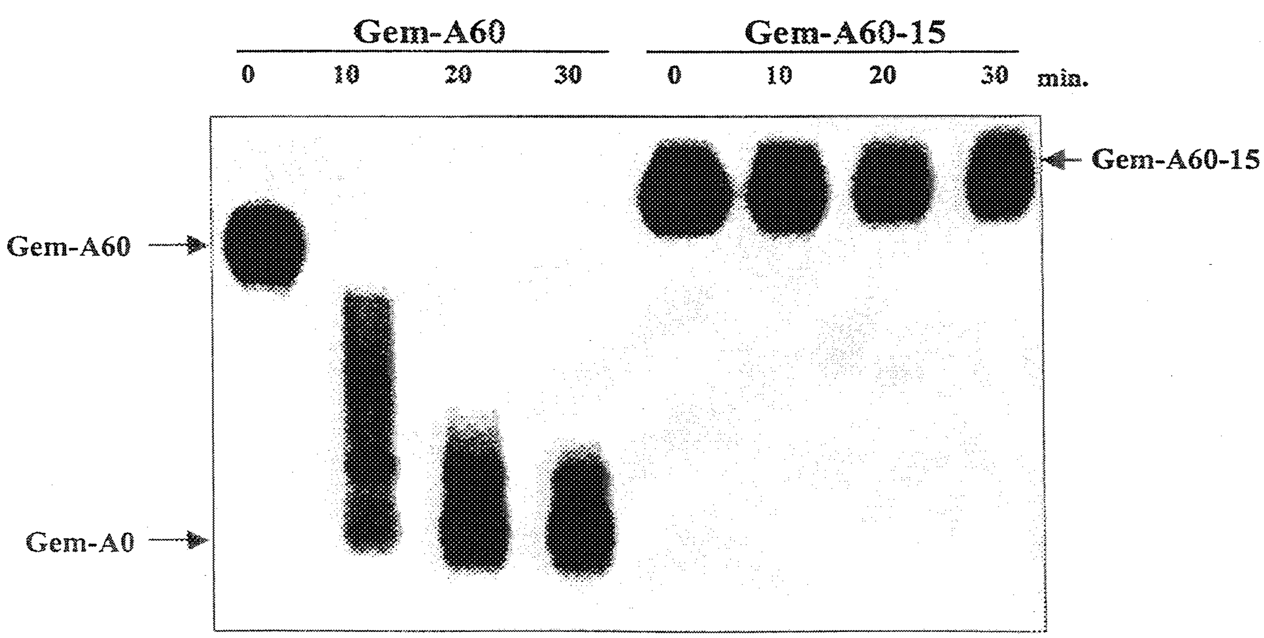

Gem-A60
Gem-A60-15
0
10
20
30
0
10
20
30
min.
Gem-A60-15
Gem-A60
Gem-A0

FIG. 2A

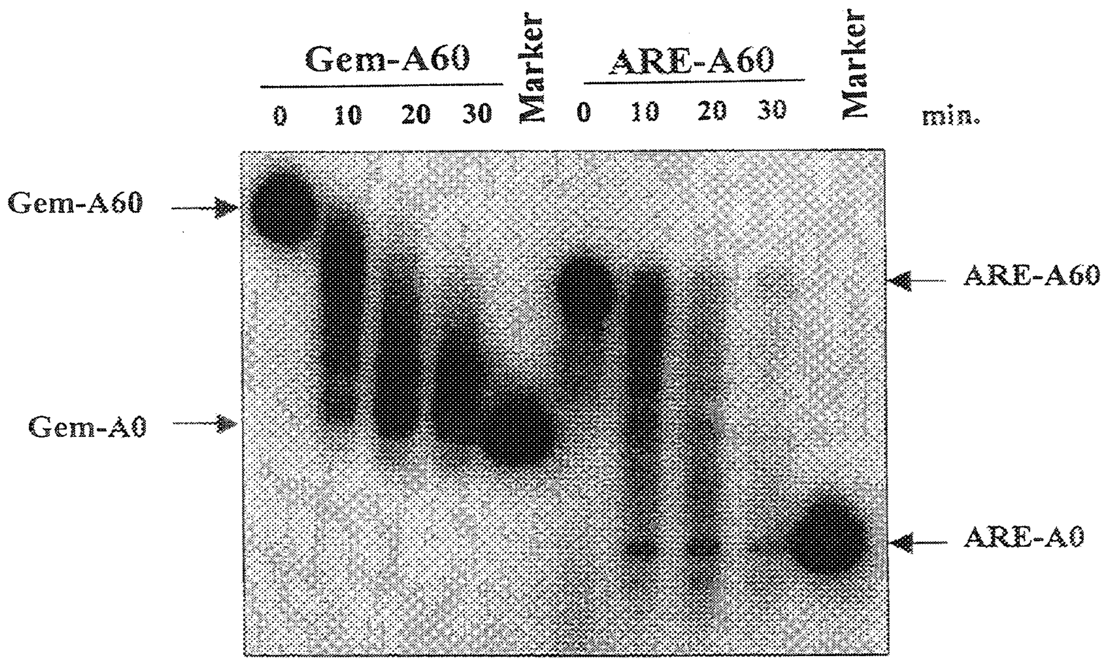

Gem-A60
Marker
ARE-A60
Marker
0 10 20 30
0 10 20 30
min.
Gem-A60
Gem-A0
ARE-A60
ARE-A0

FIG. 2B

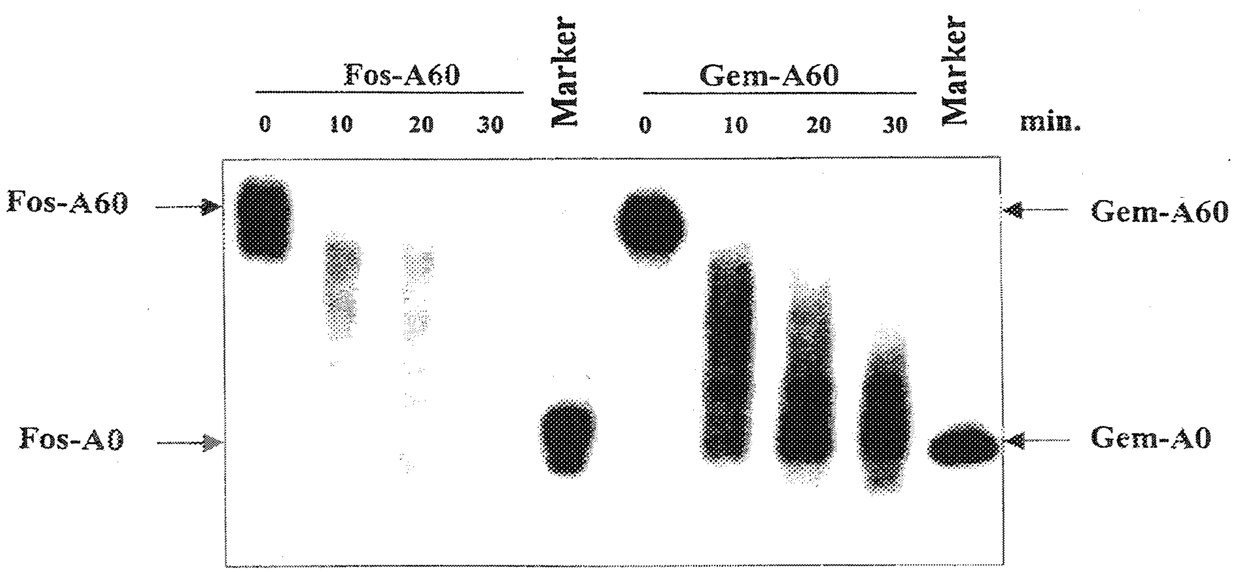
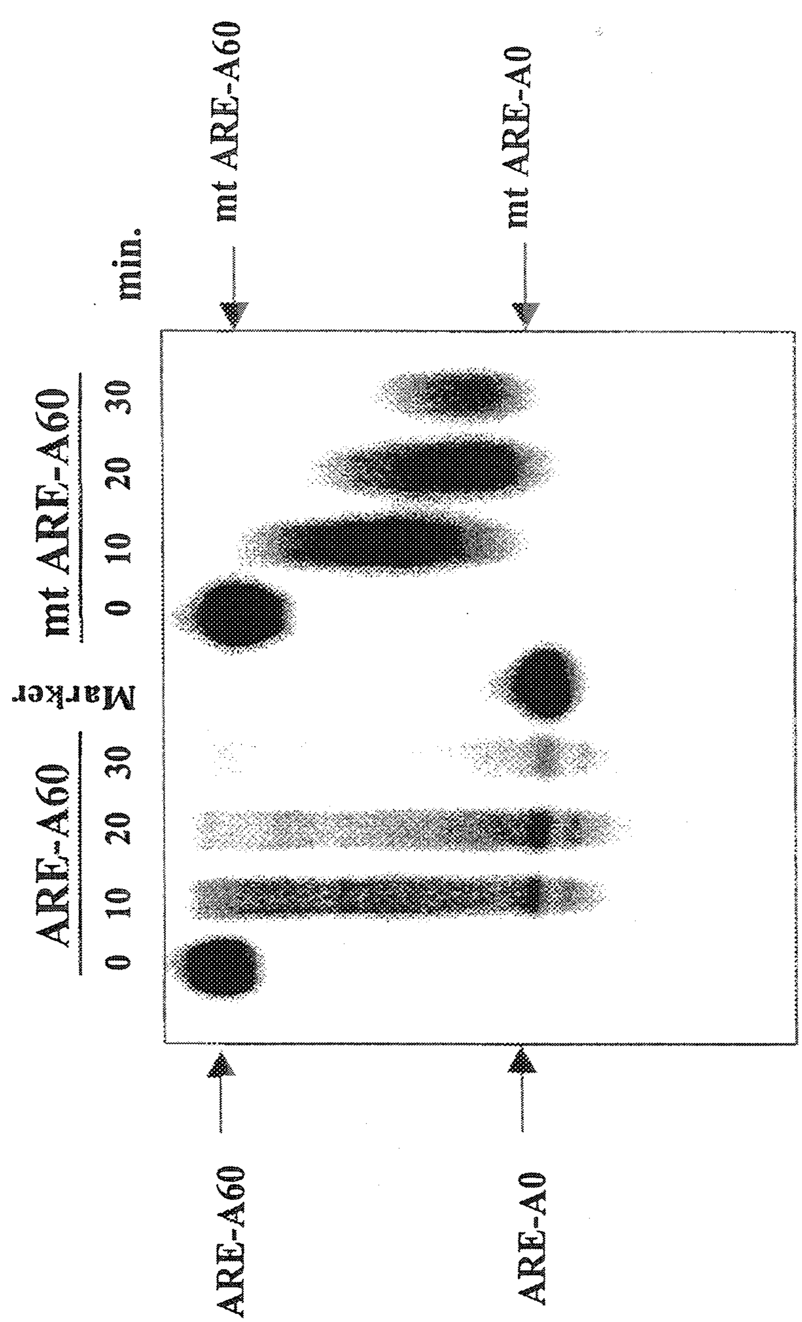

Fos-A60
Marker
Gem-A60
Marker
0 10 20 30
0 10 20 30
min.
Fos-A60
Fos-A0
Gem-A60
Gem-A0

FIG. 2C


ARE-A60
0 10 20 30
Marker
mt ARE-A60
0 10 20 30
min.
mt ARE-A60
mt ARE-A0
ARE-A60
ARE-A0

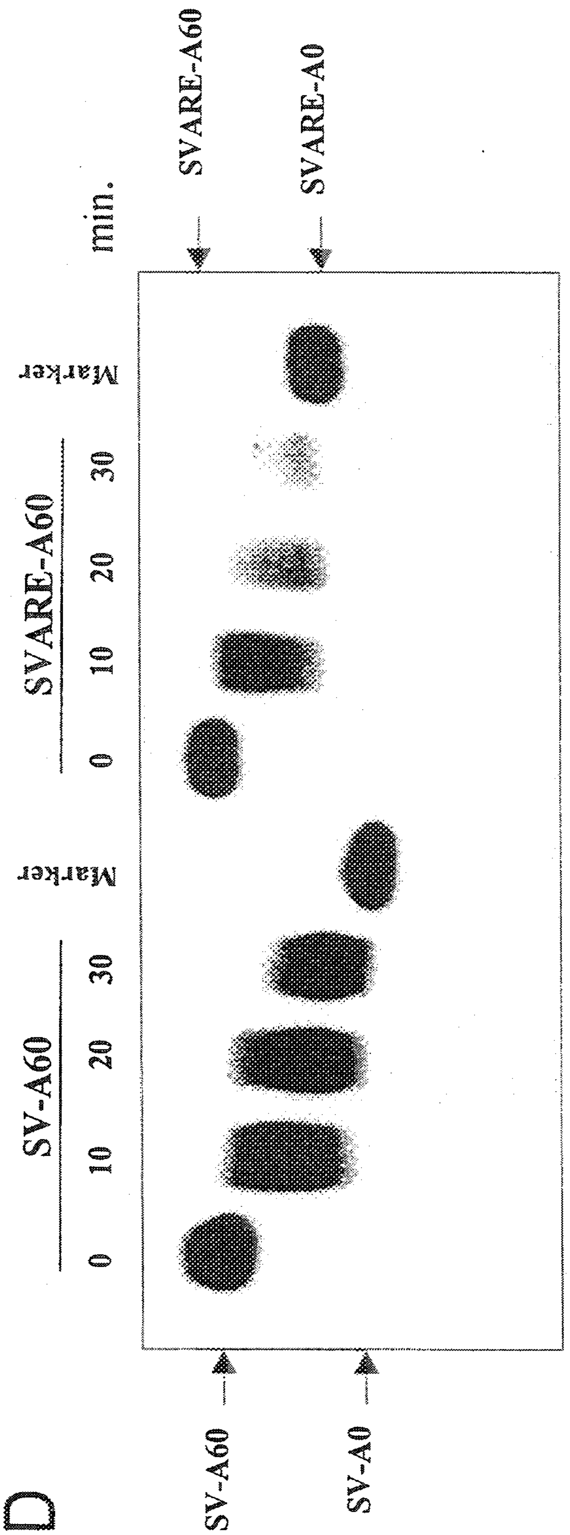

SV-A60
0
10
20
30
Marker
SVARE-A60
0
10
20
30
Marker
min.
SVARE-A60
SVARE-A0
SV-A60
SV-A0

FIG. 2D

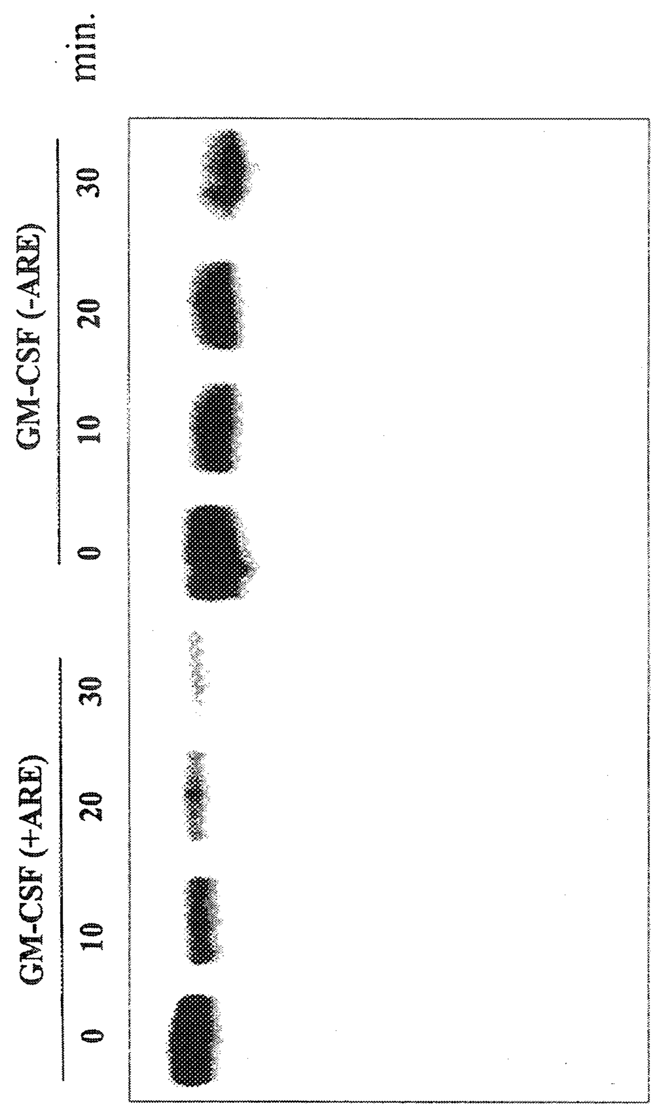

GM-CSF (+ARE)
0
10
20
30
GM-CSF (-ARE)
0
10
20
30
min.

FIG. 2E

FIG. 3A

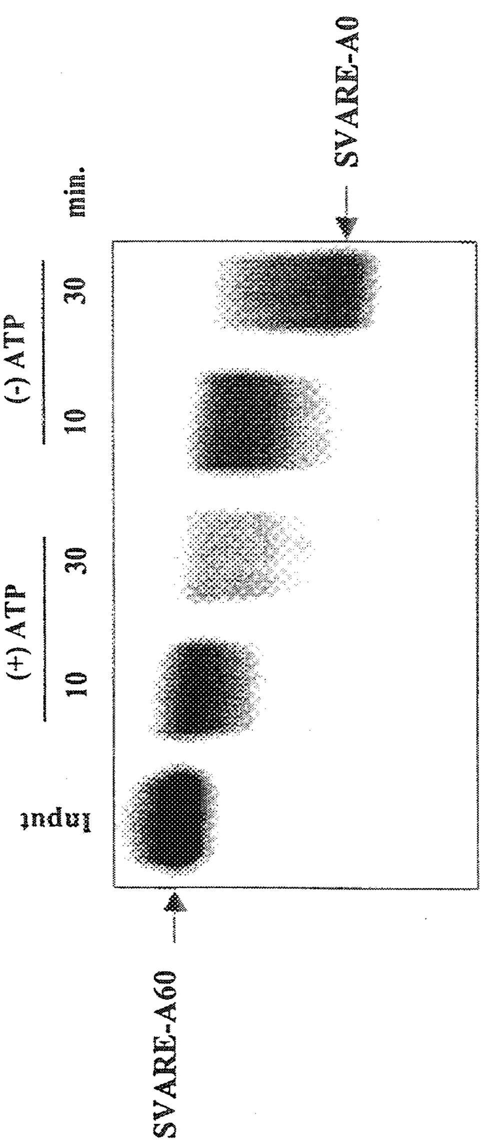

Input
(+) ATP
10
30
(-) ATP
10
30
min.
SVARE-A60
SVARE-A0

FIG. 3B

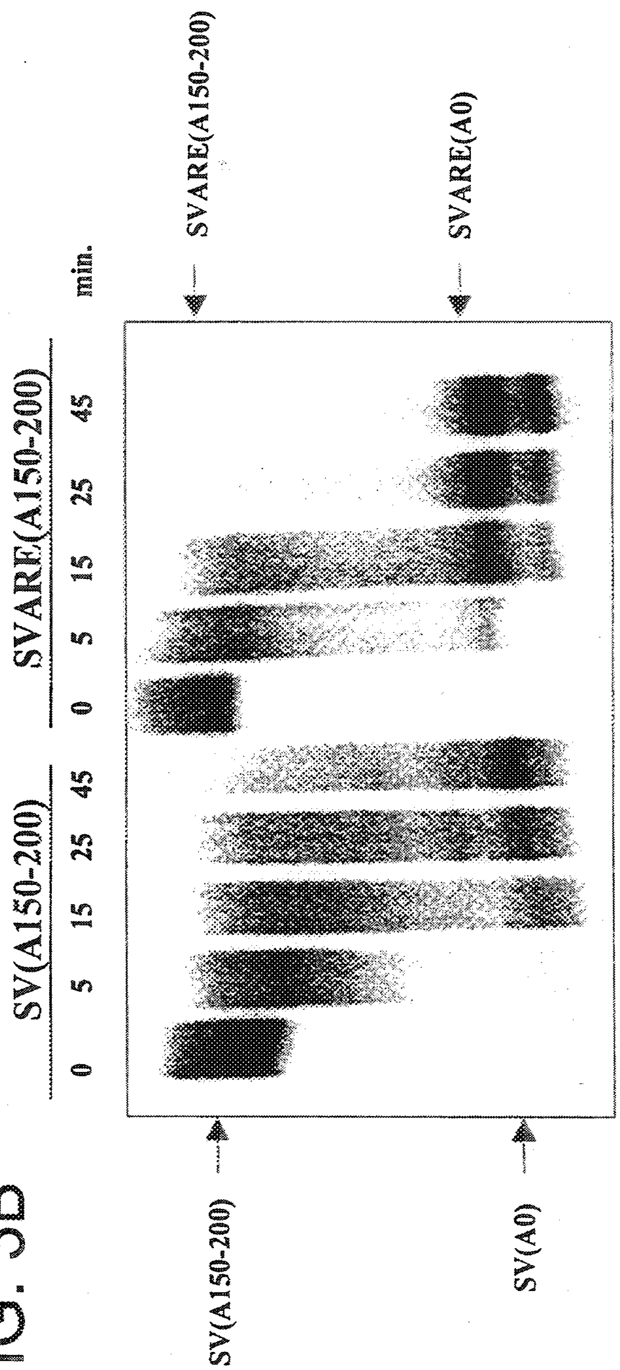

SV(A150-200)
0
5
15
25
45
SVARE(A150-200)
0
5
15
25
45
min.
SV(A150-200)
SV(A0)
SVARE(A150-200)
SVARE(A0)

FIG. 4B

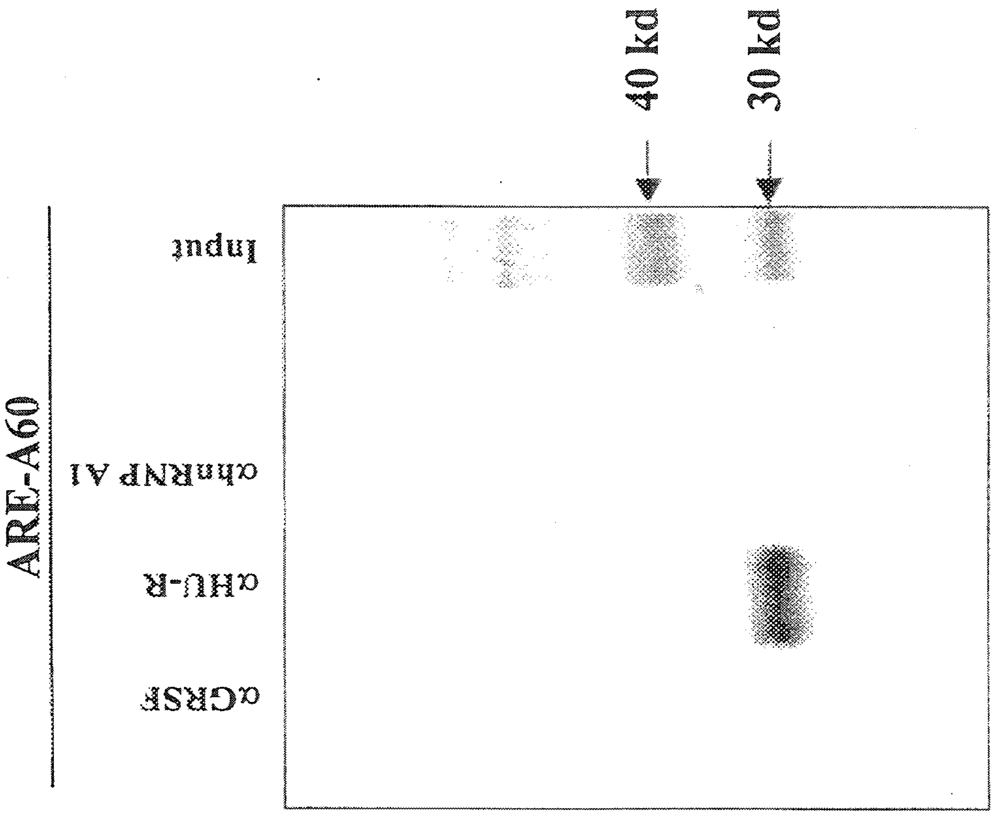

ARE-A60
αGRSF
αHU-R
αhnRNP A1
Input
40 kd
30 kd

FIG. 4A

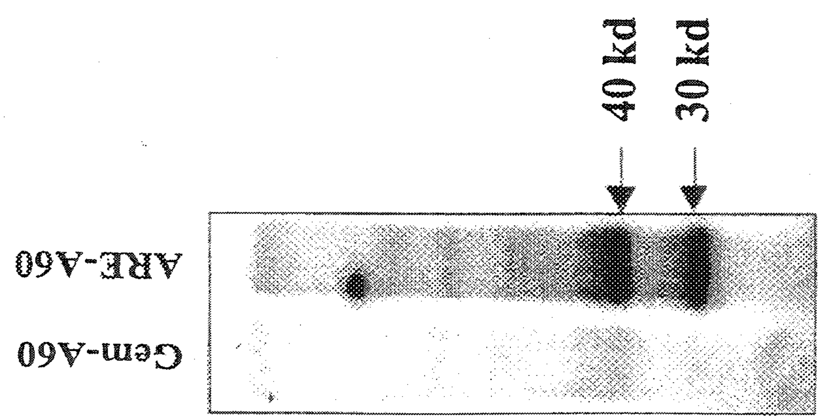

Gem-A60
ARE-A60
40 kd
30 kd

FIG. 5A

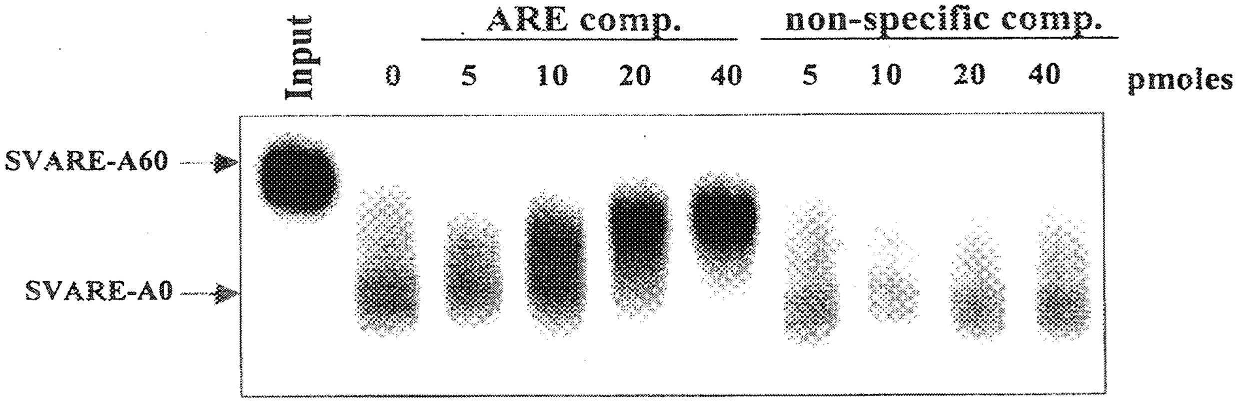


FIG. 5B

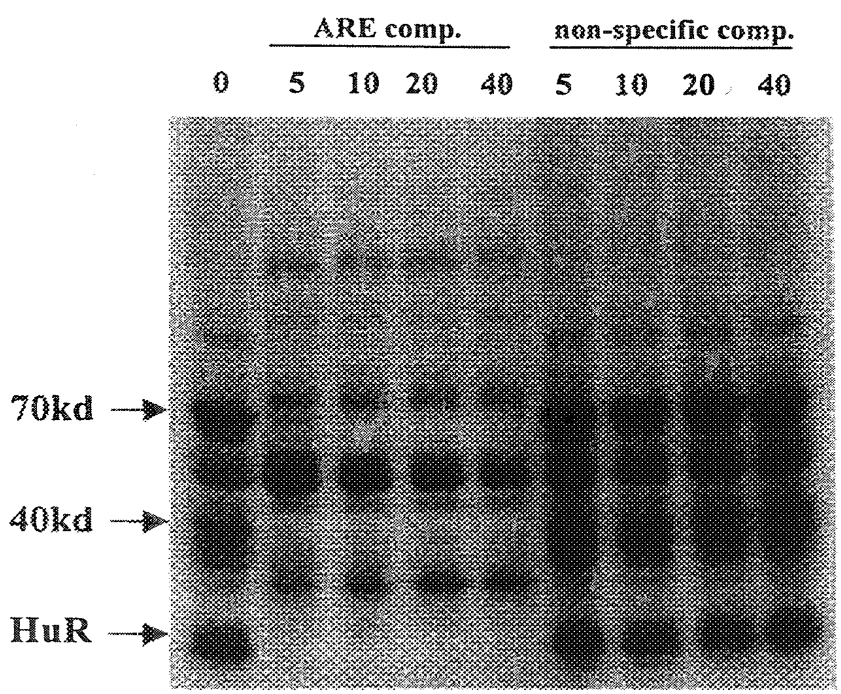


FIG. 5C

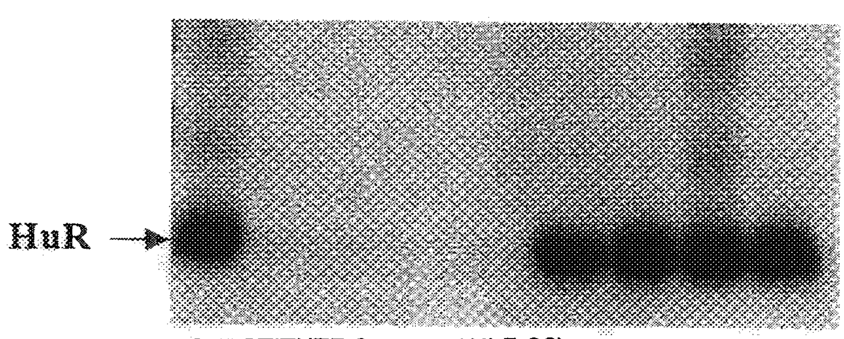


FIG. 6A

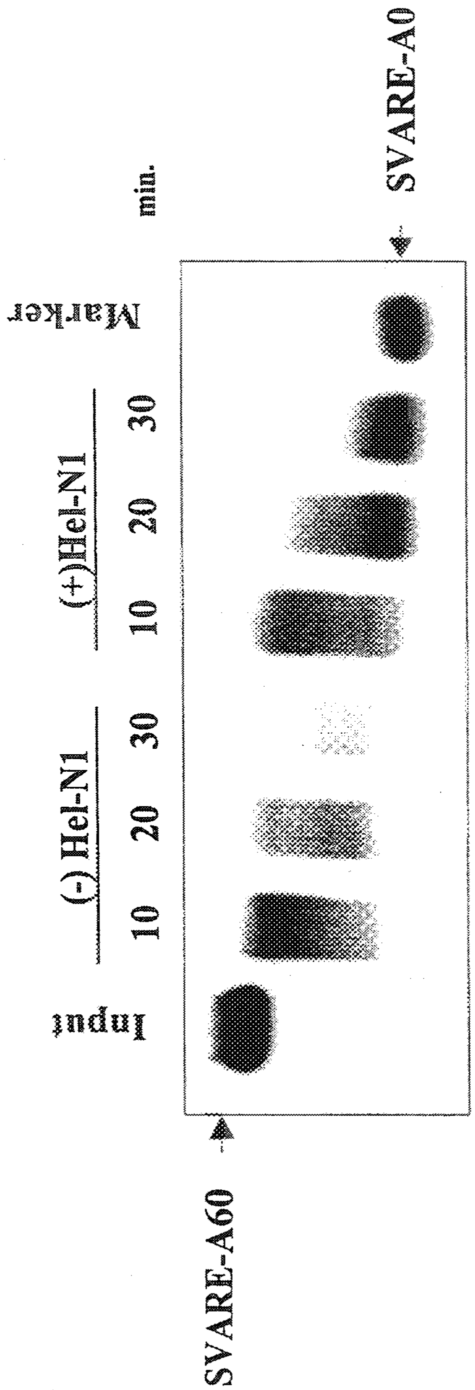

Input
(-) Hel-N1
10 20 30
(+)Hel-N1
10 20 30
Marker
min.
SVARE-A60
SVARE-A0

FIG. 6B

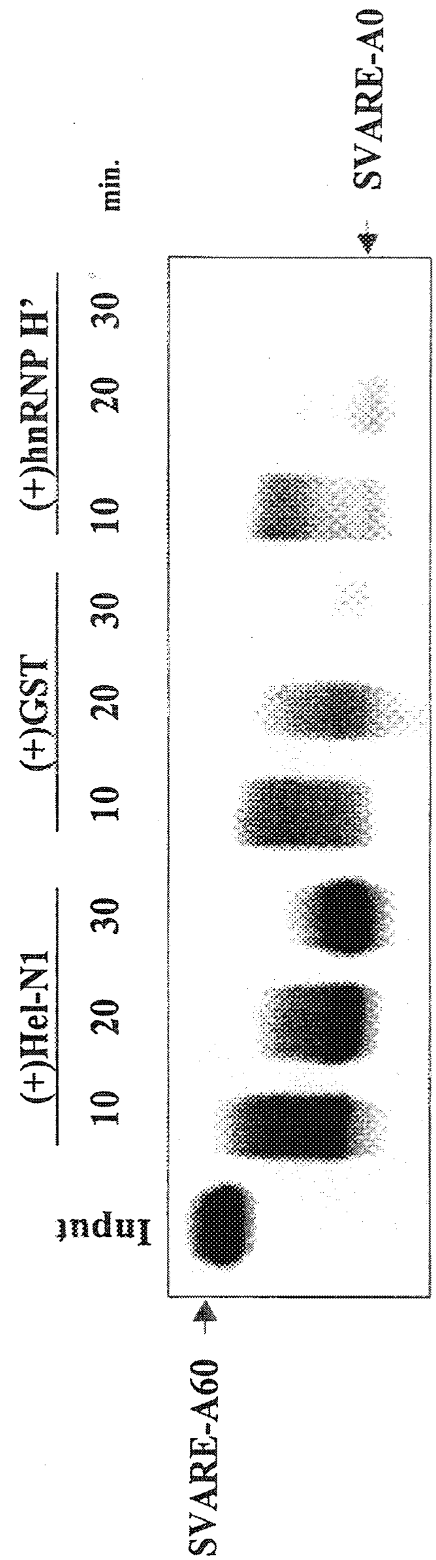

Input
(+)Hel-N1
10 20 30
(+)GST
10 20 30
(+)hnRNP H'
10 20 30
min.
SVARE-A60
SVARE-A0

FIG. 6C

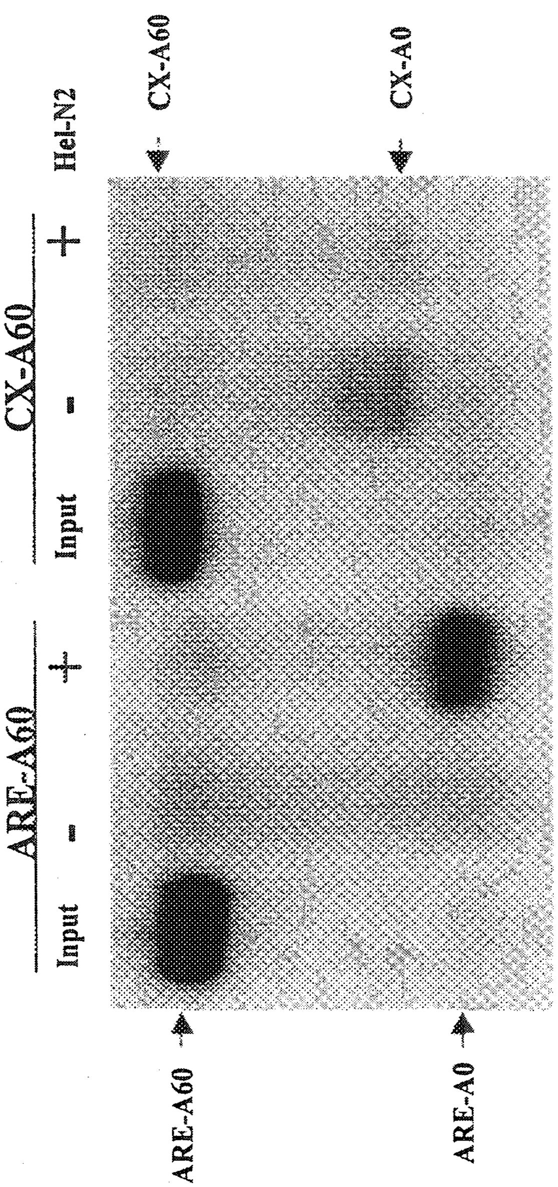
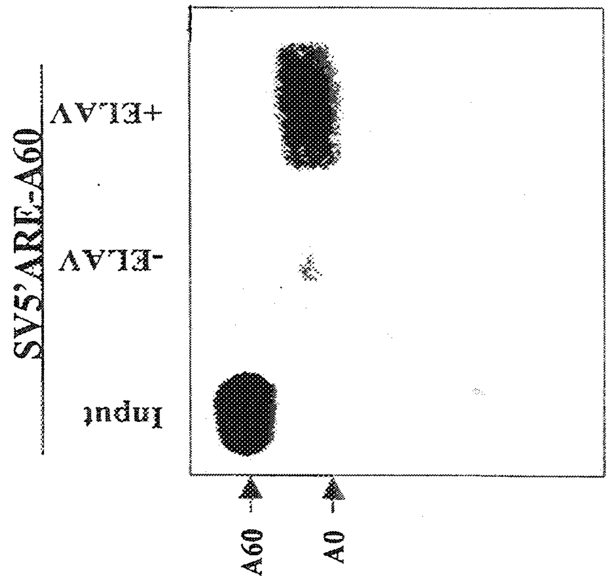

ARE-A60
Input
-
+
CX-A60
Input
-
+
Hel-N2
CX-A60
CX-A0
ARE-A60
ARE-A0

FIG. 6D


SV5'ARE-A60
Input
-ELAV
+ELAV
A60
A0

SEQUENCE IDS: 601-1-088

5'-ATTTAGGTGACACTATAGAATACACATTATTTATTATTTATTTATTATTTATTT ATTTA-3' (SEQ ID NO:1)

5'-ATTTAGGTGACACTATAGAATACACGTTAGTATTCATTTGTTTACTATTGATTT CTTTA-3' (SEQ ID NO:2)

5'-ATTTAGGTGACACTATAGAATACACAAATTTTATTGTGTTTTTAATTTATTTAT TAAGATGGATTCTC-3' (SEQ ID NO:3)

5'-ATTATTTATTATTTATTTATTATTTATTATTTA (SEQ ID NO:4)

5'-ATTTAGGTGACACTATAGAATACACCCCAACGGGCCCTCCCTCCCCTCCTTGC ACCATCATCGCATCACG (SEQ ID NO:5)

5'AUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUA (SEQ ID NO:6)

5'-GUCACGUGUCACC (SEQ ID NO:7).

5'-AGCTA$_{60}$TATTGAGGTGCTCGAGGT (SEQ ID NO:8)

5'-CATACGATTTAGGTGACACTATAG (SEQ ID NO:9)

5'-ACCTCGAGCACCTC (SEQ ID NO:10)

5'-AGTTAAATAAAT (SEQ ID NO:11)

AUUUA (SEQ ID NO: 12)

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(57) Abstract

Compounds which induce degradation of mRNA which contains one or more mRNA instability sequences are provided for use as pharmaceuticals, e.g. for use in the prophylaxis or treatment of diseases and medical conditions in general having an etiology associated with the increased or prolonged stability of mRNAs which contain one or more mRNA instability sequences, and which on prolonged or inappropriate expression typically give rise to undesirable effects, e.g. cancer cell growth or an unwanted inflammatory response.

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COMPOUNDS WHICH AFFECT mRNA STABILITY AND USES THEREFOR

This invention relates to biologically active compounds and to their use in the treatment and prophylaxis of disease. In particular the invention relates to compounds which affect the stability of mRNA which contain one or more mRNA instability sequences.

Recently, it has become increasingly apparent that the regulation of RNA half-life plays a critical role in the tight control of gene expression and that mRNA degradation is a highly controlled process. RNA instability allows for rapid up- or down-regulation of mRNA transcript levels upon changes in transcription rates. A number of critical cellular factors, e.g. transcription factors such as c-myc, or gene products which are involved in the host immune response such as cytokines, are required to be present only transiently to perform their normal functions. Transient stabilisation of the mRNAs which code for these factors permits accumulation and translation of these messages to express the desired cellular factors when required; whereas, under non-stabilised, normal conditions the rapid turnover rates of these mRNAs effectively limit and "switch off" expression of the cellular factors. However, abnormal regulation of mRNA stabilisation can lead to unwanted build up of cellular factors leading to undesirable cell transformation, e.g. tumour formation, or inappropriate and tissue damaging inflammatory responses.

Although the mechanisms which control mRNA stability are far from understood, sequence regions have been identified in a number of mRNAs, which appear to confer instability on the mRNAs which contain them. These sequence regions are referred to herein as "mRNA instability sequences". For example, typical mRNA instability sequences are the AREs (AU rich elements), which are found in the 3'UTR (3' untranslated region) of certain genes including a number of immediate early genes and genes coding for inflammatory cytokines, e.g. IL-1β and TNFα.

Kastelic et al. (CYTOKINE, Vol. 8, No. 10, (October), 1996: pp751-761) have reported the finding that radicicol analog A, if added to THP-1 cells activated by IFN-γ and LPS, not only inhibited the secretion of IL-1β but also induced an extremely rapid degradation of IL-1β, IL-6

and TNF-α mRNA to undetectable levels in 5-8 h, and that this mRNA degradation appears to be mediated through AU-rich regions present in the 3' untranslated regions of the RNAs which code for these cytokines.

Previously, novel Radicicol analogs (including radicicol analog A), processes for their preparation and their pharmaceutical use were described in European patent application EP 0606044 A, together with known compounds including radicicol, O-methyl radicicol, and the related compound zearelenone and certain analogs of zearelenone. The radicicol analogs and known compounds are described in EP 0606044 A to be useful for the treatment of disorders with an etiology associated with or comprising excessive cytokine release, particularly IL-1β release, such as rheumatoid arthritis, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma.

We have now found that there are other compounds in addition to radicicol analog A which induce degradation of mRNAs and that such compounds may be used for treatment of diseases and medical conditions which involve increased or prolonged stability and expression of such mRNAs. Moreover we have found that radicicol analog A may be used generally to induce degradation of mRNAs besides IL-1β, IL-6 and TNF-α mRNAs.

Accordingly the present invention provides a compound which induces degradation of mRNA which contains one or more mRNA instability sequences for use as a pharmaceutical, provided the compound is not radicicol analog A.

In a further aspect the invention provides a method for the prophylaxis or treatment of a disease or medical condition having an etiology associated with the increased stability of mRNA which contains one or more mRNA instability sequences, comprising administering to a human or animal patient an effective amount of a compound which induces degradation of the mRNA, provided that the compound is not radicicol analog A when the disease or medical condition is one with an etiology associated with or comprising excessive cytokine release, particularly IL-1β

release, such as rheumatoid arthritis, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma.

In a yet further aspect the invention provides use of a compound which induces degradation of mRNA which contains one or more mRNA instability sequences, for the preparation of a medicament for use in the treatment or prophylaxis of a disease or medical condition having an etiology associated with the increased stability of mRNA which contains one or more mRNA instability sequences, provided that the compound is not radicicol analog A when the disease or medical condition is one with an etiology associated with or comprising excessive cytokine release, particularly IL-1β release, such as rheumatoid arthritis, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma.

The invention also provides a method for inducing degradation of mRNA in a patient, which comprises administering an effective amount of a compound which induces mRNA degradation to the patient, wherein the mRNA contains an mRNA instability sequence, provided that the compound is not radicicol analog A when the mRNA is mRNA coding for IL-1β, IL–6 or TNF-α.

Further the invention provides use of a compound which induces mRNA degradation in the preparation of a medicament for use in inducing degradation of mRNA which contains a mRNA degradation sequence in a patient, provided that the compound is not radicicol analog A when the mRNA is mRNA coding for IL-1β, IL–6 or TNF-α.

The present invention further provides the use of a radicicol analog for preparation of a medicament for treatment of a cancer and/or malignant disease.

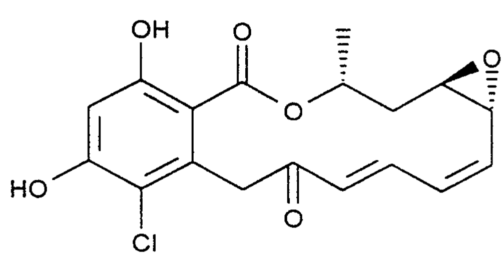
The present invention also provides a method for the prophylaxis or treatment of a cancer and/or malignant disease comprising administering to a patient an effective amount of a radicicol analog.

Any compound which induces degradation of mRNA which contains a mRNA instability sequence is potentially of interest for use in the present invention. Compounds which induce degradation of mRNA which contains a mRNA instability sequence are hereinafter referred to as Compounds for use in the invention. Such compounds include radicicol analogs, in particular radicicol analog A or radicicol; for instance, as described in EP 0606044.

Our copending British patent application no. 9828709.7 describes a reporter gene assay for the identification of compounds which destabilise mRNA. In this assay test compounds are contacted with a DNA expression system which in the absence of the compound is capable of expressing a protein having a detectable signal, and wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence. The detectable signal is measured in the presence of the test compound and the result obtained is compared with a control. Compounds which destabilise mRNAs induce degradation of the mRNA which codes for the detectable signal leading to a decrease in the magnitude of the detectable signal obtained in the reporter gene assay.

Preferred compounds for use in the present invention include compounds which may be identified as inducers of mRNA instability using the reporter gene assay as described above and as described in more detail in the above mentioned British patent application no. 9828709.7 and as hereinafter described in the Examples. Particular examples of compounds for use in the present invention include radicicol and the radicicol analogs.

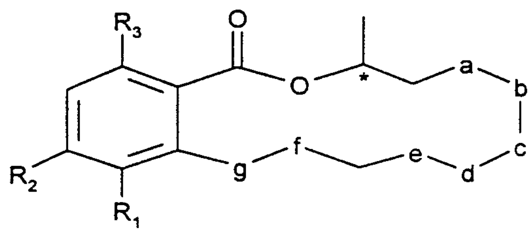
Radicicol, the compound of formula I



I

has been known for many years as a natural compound, e.g. as a metabolite of the microorganism *Monosporium bonorden*, and was described initially as having antibiotic properties (Delmotte, Nature 171, 344 (1953)).

A particular class of radicicol analogs which includes Compounds for use in the invention are compounds of formula II



II

wherein

R_1 is H, OH, halogen, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-;

R_2 is OH, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-;

R_3 is H, OH, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-;

-a-b- is -CHR_7-CHR_8- or <u>cis</u> or <u>trans</u> -CR_7=CR_8-,

wherein R_7 and R_8 are the same or different and are H, OH, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-, or

-a-b- is -CHR_7-CHR_8- and R_7 and R_8 together with O form an epoxide bridge;

c is >CH-OH, >C=O or >CH_2;

-d-e- is -CHR_7-CHR_8- or <u>cis</u> or <u>trans</u> -CR_7=CR_8-,

wherein R_7 and R_8 are the same or different and are H, OH, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-, and

-f-g- is -CH_2-CH_2-, <u>cis</u> or <u>trans</u> -CH=CH-, or -C(O)-CH_2-,

and pharmaceutically acceptable salts thereof and physiologically-hydrolysable and -acceptable esters thereof.

The carbon atom marked with an asterisk (*) in formula II is an asymmetric carbon atom. The carbon atoms at a, b, c or d may also be asymmetric carbon atoms dependent upon the particular substituents present at these positions. Asymmetric carbon atoms at these positions

may have the R- or S-configuration or the radicicol analog may comprise any mixture of the optical isomers thereof. Preferred isomers include those specifically described hereinafter.

Halogen or halo as used herein refers to F, Cl, Br or I unless otherwise indicated, preferably Cl.

A particular subset of the compounds of formula II are those in which one of -a-b- or -d-e- is $-CHR_7-CHR_8-$ and the other is cis- or trans- $-CR_7=CR_8-$, wherein R_7 and R_8 are the same or different and are H, OH, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-, and c is >CH-OH or >C=O, and wherein R_1, R_2, R_3 and -f-g- are as defined above.

Particular significances for the variable substituents and moieties of the radicicol analogs of formula II are as follows:

Preferably R_1 and R_3 are the same or different and are H, -OH, MeO- or Me-COO-. Preferably R_2 is -OH, MeO- or MeCOO-. More preferably R_1 is H or MeO; R_2 is MeO, and R_3 is OH or MeO.

Preferably -a-b- is cis- or trans- $-CR_7'=CR_8'-$, wherein R_7' and R_8' are the same or different and are H, OH, MeO- or Me-COO-. More preferably -a-b- is cis- or especially trans- -CH=CH-.

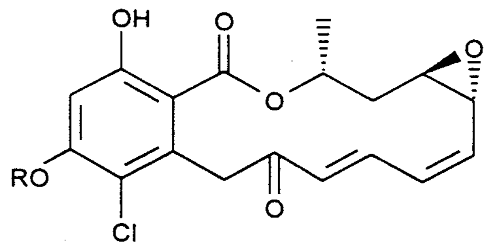
Preferably -d-e- is $-CHR_7'-CHR_8'-$, wherein R_7' and R_8' are as defined above. More preferably -d-e- is $-CH_2-CH_2-$ or especially -CHOH-CHOH-, wherein the OH groups may be in free or protected form.

Most preferably -f-g- is trans- -CH=CH-.

Preferably the asymmetric carbon atoms of the compounds of the invention all have the S-configuration.

Particular radicicol analogs of formula II for use in the invention are analogs of formula II in which R_1 is H or methoxy, R_2 is methoxy, R_3 is OH, -a-b- is cis- or trans- -CH=CH-, c is CHOH or C=O, -d-e- is -CHOH-CHOH- and -f-g- is trans- -CH=CH-; in free form or base salt form or in the form of a physiologically-hydrolysable and -acceptable ester.

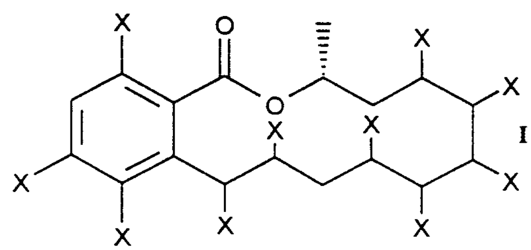
Particular radicicol analogs for use in the present invention include radicicol and O-lower alkyl radicicols, i.e. the compounds of formula I'



I'

where R is H or C_1-C_4 lower alkyl, e.g. methyl, and pharmaceutically acceptable salts thereof and physiologically-hydrolysable and -acceptable esters thereof.

For the purposes of the present description a radicicol analog is a compound having the characteristic bicyclic ring structure of radicicol, i.e. the structure of formula I'',



I'

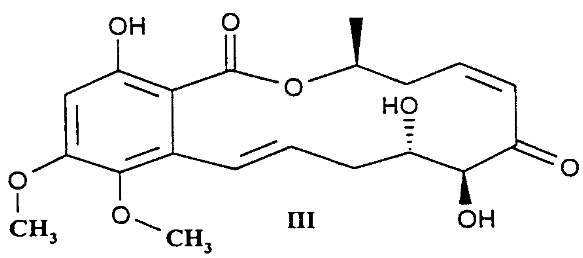
wherein the X groups are separately H or substituents, the 14-membered lactam ring may additionally comprise one or more, e.g. two, ethylenically unsaturated bonds and at least one of the X substituents of the lactam ring may comprise an oxy (=O), or (with an adjacent X substituent) an epoxide substituent, and pharmaceutically acceptable salts thereof and physiologically-hydrolysable and -acceptable esters thereof.

Radicicol analogs which comprise -OH substituents may also exist in the form of pharmaceutically acceptable esters, and the use of such is included within the scope of the invention. Pharmaceutically acceptable esters are preferably prodrug ester derivatives, such being

convertible by solvolysis or under physiological conditions to the free radicicol analog. Preferred pharmaceutically acceptable prodrug esters of the are those derived from a carboxylic acid, a carbonic acid monoester or a carbamic acid, advantageously esters derived from an optionally substituted lower alkanoic acid or an arylcarboxylic acid.

Radicicol analogs may also exist in the form of pharmaceutically acceptable salts, and the use of such is included within the scope of the invention. Pharmaceutically acceptable salts represent acid addition salts with conventional acids, for example, mineral acids, e.g., hydrochloric acid, sulfuric or phosphoric acid, or organic acids, for example, aliphatic or aromatic carboxylic or sulfonic acids, e.g., acetic, propionic, succinic, glycolic, lactic, malic, tartaric, citric, ascorbic, maleic, fumaric, hydroxymaleic, pyruvic, pamoic, methanesulfonic, toluenesulfonic, naphthalenesulfonic, sulfanilic or cyclohexylsulfamic acid; also amino acids, such as arginine and lysine. For compounds of the invention having acidic groups, for example, an acidic -OH group, pharmaceutically acceptable salts also represent metal or ammonium salts, such as alkali metal or alkaline earth metal salts, e.g., sodium, potassium, magnesium or calcium salts.

EP 0606044 A describes the isolation and characterisation of the radicicol analog of formula III,



III

hereinafter referred to as radicicol analog A, which was first identified as a natural product isolated from a strain of pycnidia imperfect fungi (F/87-250904) deposited on 6 November 1991 with the ARS Patent Culture Collection, US Dept. of Agriculture, Northern Regional Research Centre, Peoria, Illinois, USA under the provisions of the Budapest Treaty as deposit NRRL 18919.

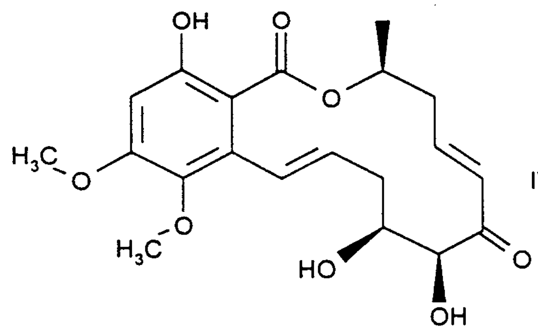
Radicicol analog A is a particularly preferred radicicol analog for use in the present invention. Radicicol analog A also serves as a valuable starting material for synthesis of other radicicol analogs for use in the present invention. Alternatively EP 0606044 A describes the de novo synthesis of radicicol analogs starting from readily available starting materials.

Novel Radicicol analogs, processes for their preparation and their pharmaceutical use are described in European patent application EP 0606044 A, together with known compounds including radicicol, O-methyl radicicol, and the related compound zearelenone and certain analogs of zearelenone. The radicicol analogs and known compounds are described in EP 0606044 A to be useful for the treatment of disorders with an aetiology associated with or comprising excessive cytokine release, particularly IL-1β release, such as rheumatoid arthritis, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma..

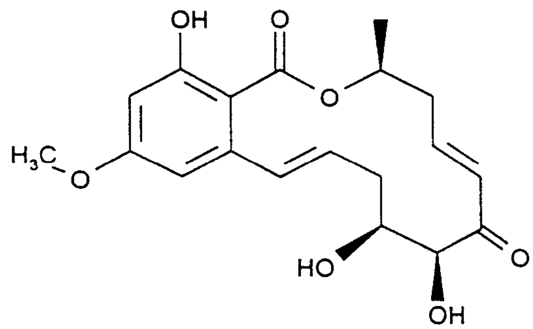
The disclosure of EP 0606044 relating to the isolation of radicicol analog A from the fungal strain F/87-250904, the synthesis of semi-synthetic radicicol analogs from radicicol analog A and the de novo synthesis of radicicol analogs, is specifically incorporated by reference in the teaching of the present application.

Surprisingly it has now been found that radicicol, radicicol analogs, zearelenone and zearelenone analogs (hereinafter collectively referred to as radicicol analogues), such as those described in EP 0606044 A, are useful for treatment of certain forms of cancer and malignant diseases.

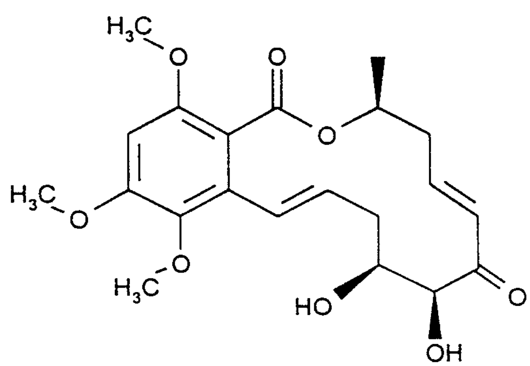
Particularly preferred radicicol analogs for use in the invention include compounds of formula II in which -a-b- is trans- -CH=CH-, e.g. the compounds of formulae IV, V and VI



IV

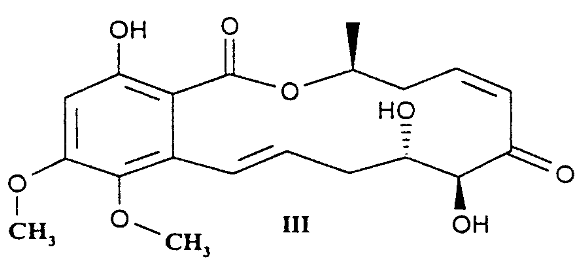


V

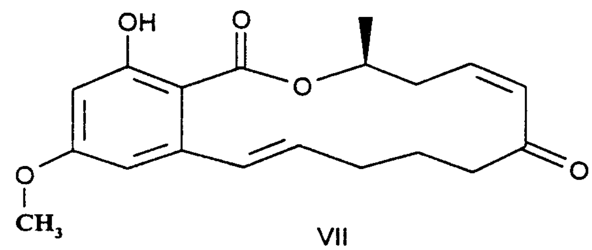


VI

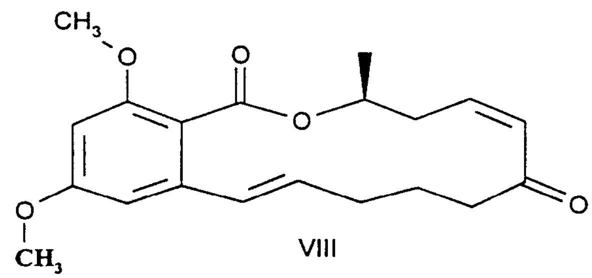
Particularly preferred radicicol analogs for use in the invention include compounds of formula II in which -a-b- is trans- -CH=CH-, e.g. the compounds of formulae III, VII and VIII.



III



VII



VIII

The present invention may be used in the prophylaxis or treatment of diseases and medical conditions in general having an etiology associated with the increased or prolonged stability of mRNAs which contain one or more mRNA instability sequences, and which on prolonged or inappropriate expression typically give rise to undesirable effects, e.g. cancer cell growth or an unwanted inflammatory response.

mRNA instability sequences have been identified in the UTRs, in particular the 3' UTRs, of a large number of transiently expressed genes including genes for cytokines, chemokines, nuclear transcription factors, protooncogenes, immediate early genes, cell cycle controlling genes, oxygenases, and genes involved in and controlling of apoptosis. The natural RNA sequences which comprise the mRNA instability sequences are alternatively referred to as adenylate/uridylate (AU)-rich elements, or AREs. Transiently expressed genes which contain mRNA instability sequences include, for example, the genes coding for GM-CSF, c-*fos*, c-*myc*, c-*jun*, *krox*-20, *nur*-77, *zif268*, β-IFN, uPA, IL-1, IL-3, TNF-α, MCP1, syn1, β_2-AR, E-selectin, VCAM-1, ICAM-1, P-glycoproteins (MDR), MRPs, Pγh1 (pf mdr), COX II, metalloproteinases (MMPs), bcl-2 and MIP-2α.

The following publications include extensive discussion of mRNA instability sequences and AREs, the sequences motifs which they contain and (minimum) sequence requirements for mRNA destabilisation, as well as identifying a number of mRNA instability sequences and the genes which contain them:

Shaw & Kamen, Cell, Vol. 46, 659-667, August 29 1986 (GM-CSF);

Shyu et al., Genes & Development, 5:221-231 (1991) (c-*fos*);

Sachs, Cell, Vol. 74, 413-421, August 13 1993 (Review. "Messenger RNA Degradation in Eukaryotes");

Chen et al., Mol. Cell. Biol., Jan 1994, p 416-426 (c-*fos*);

Akashi et al., Blood, Vol. 83, No. 11, (June 1), 1994: pp 3182-3187 (GM-CSF etc.);

Nanbu et al., Mol. Cell. Biol., July 1994, p. 4920-4920 (Upa);

Stoecklin et al., J. Biol. Chem., Vol. 269, No. 46, November 18 1994, pp 28591-28597 (IL-3);

Lagnado et al., Mol. Cell. Biol., Dec. 1994, p. 7984-7995 (general);

Zhang et al., Mol. Cell. Biol., Apr. 1995, p. 2231-2244 (yeast);

Zubiaga et al., Mol. Cell. Biol., Apr. 1995, p. 2219-2230 (general);

Winstall et al., Mol. Cell. Biol., July 1995, p. 3796-3804 (c-*fos*, GM-CSF);

Chen et al., Mol. Cell. Biol., Oct. 1995, p. 5777-5788 (c-*fos*, GM-CSF);

Chen et al., TIBS 20 November 1995, 465-470 (review);

Levy et al., J. Biol. Chem., Vol. 271, No. %, February 2 1996, pp. 2746-2753 (VEGF);

Kastelic et al., Cytokine, Vol. 8, No. 10 (October), 1996: pp751-761;

Crawford et al., J. Biol. Chem., Vol. 272, No. 34, August 22 1997, pp. 21120-21127 (TNF-α);

Xu et al., Mol. Cell. Biol., Aug. 1997, Vol. 18, No. 8, p. 4611-4621 (general);

Danner et al., J. Biol. Chem., Vol.273, No. 6, February 6 1998, pp. 3223-3229 (human β_2-Adrenergic Receptor);

Lewis et al., J. Biol. Chem., Vol. 273, No. 22, May 29 1998, pp. 13781-13786 (TNF-α).

As described in the above publications mRNA instability sequences often contain one or more copies of sequence motifs, e.g. selected from: AUUUA, UAUUUAU, UUAUUUA(U/A)(U/A), and AUUUAUUUA. Such sequence motifs are typically in genes between the stop codon and the poly A signal and may associated with appropriate flanking

sequences and may interact in combination with other sequences, e.g. present in the 5' UTR and e.g. with instability motifs present in the coding region.

The present invention may be used in connection with diseases and medical conditions associated with any of the genes mentioned above or described in the listed publications, which comprise mRNA instability sequences.

Examples of diseases and medical conditions which may be treated or prevented by use of the present invention include: cancers e.g. of the colon, breast, lung etc., acute and chronic inflammation, autoimmune diseases, respiratory diseases, infectious diseases and transplant rejection.

The compounds for use in the invention have valuable pharmacological properties. In particular compounds for use in the invention have valuable properties as inducers of degradation of mRNAs which contain mRNA instability sequences. The activity of compounds for use in the invention as inducers of mRNA degradation may be demonstrated by means of a reporter gene assay as hereinafter described in the Examples, or as described in more detail in our copending British patent application no. 9828709.7.

In view of their activity as inducers of degradation of mRNAs which contain mRNA instability sequences, the radicicol analogues are useful for the prophylaxis and treatment of cancers and malignant diseases which involve inappropriate build-up and expression of mRNAs, which contain mRNA instability sequences, and which code for proteins involved in the initiation, progression or persistence of cancer or malignant disease. Examples of cancer related genes, with mRNAs which contain mRNA destabilising sequences, include various oncogenes and transcription factors, e.g. c-myc, c-fos, Spl, bcl-2 and similar genes. The inappropriate or prolonged expression of such oncogenes is implicated in the initiation of certain forms of cancer, such as colon cancer, breast cancer, lung cancer etc.. Further examples of cancer related genes, with mRNAs which contain mRNA instability sequences are genes for metalloproteinase enzymes, e.g. MMP-1, MMP-2, collagenases etc., involved in tissue remodelling required for

tumour growth and metastasis invasion; cell cycle related genes such as p45/SKIP2 etc. and multidrug resistance genes, e.g. mdr-1, MRPs, etc. involved in the intrinsic or acquired multidrug resistance of some cancer cells.

Treatment with radicicol analogs advantageously leads to degradation of the mRNAs of such genes, resulting in the down-regulation or "switching off" of gene expression. Thus for example, radicicol analogs may be use for treatment and prevention of oncogene mediated cancers and malignant diseases, to treat or prevent tumour growth and metastasis invasion in general, and to prevent or reverse multidrug resistance and thereby facilitate cancer and tumour treatment with conventional, e.g. cytotoxic, anti-cancer agents.

Radicicol analogs may be tested for their activity as anti-cancer agents in cell or *in vivo* assays substantially as described below or in variants of such assays using appropriate cell lines and conditions.

Radicicol analogs exhibit, for example, inhibition of the cell growth of EGF-dependent cell lines, for example the epidermoid BALB/c mouse keratinocyte cell line (see Weissmann, B.A., and Aaronson, S.A., Cell 32, 599 (1983)) or the A431 cell line, which are recognised useful standard sources of EGF-dependent epithelial cells (see Carpenter, G., and Zendegni, J. Anal. Biochem. 153, 279-282 (1985)). In a known test method (see Meyer *et al.*, Int. J. Cancer 43, 851 (1989)), the inhibitory activity of radicicol analogs is determined, briefly, as follows: BALB/MK cells (10 000/microtitre plate well) are transferred to 96-well microtitre plates. The test compounds (dissolved in DMSO) are added in a series of concentrations (dilution series) in such a manner that the final concentration of DMSO is not greater than 1 % (v/v). After the addition, the plates are incubated for three days during which the control cultures without test compound are able to undergo at least three cell-division cycles. The growth of the MK cells is measured by means of methylene blue staining: after the incubation the cells are fixed with glutaraldehyde, washed with water and stained with 0.05 % methylene blue. After a washing step the stain is eluted with 3 % HCl and the optical density per well of the microtitre plate is measured using a

Titertek multiskan at 665 nm. IC_{50} values are determined by a computer-aided system using the formula:

$$IC_{50} = [(OD_{test} - OD_{start})/(OD_{control} - OD_{start})] \times 100.$$

The IC_{50} value in those experiments is given as that concentration of the test compound in question that results in a cell count that is 50 % lower than that obtained using the control without inhibitor. The radicicol analogs exhibit inhibitory activity in the micromolar range, for example an IC_{50} of approximately from 0.1 to 10 mM, especially from 0.4 to 4 mM.

The radicicol analogs exhibit inhibition of the growth of tumour cells also *in vivo*, as shown, for example, by the test described below: the test is based on inhibition of the growth of the human epidermoid carcinoma A431 (ATCC No. CRL 1555; American Type Culture Collection, Rockville, Maryland, USA; see Santon, J.B., *et al.*, Cancer Research 46, 4701-4705 (1986) and Ozawa, S., *et al.*, Int. J. Cancer 40, 706-710 (1987)), which is transplanted into female BALB/c nude mice (Bomholtgard, Denmark). That carcinoma exhibits a growth that correlates with the extent of the expression of EGF-receptor. In the experiment, tumours having a volume of approximately 1 cm^3 cultured *in vivo* are surgically removed from experimental animals under sterile conditions. The tumours are comminuted and suspended in 10 volumes (w/v) of phosphate-buffered saline. The suspension is injected s.c. (0.2 ml/mouse in phosphate-buffered saline) into the left flank of the animals. Alternatively, 1×10^6 cells from an *in vitro* culture can be injected in 0.2 ml of phosphate-buffered saline. Treatment with test compounds is started 5 or 7 days after the transplant, when the tumours have reached a diameter of 4-5 mm. The test compound in question is administered (in different doses for different animal groups) once a day for 15 successive days. The tumour growth is determined by measuring the diameter of the tumours along three axes that are perpendicular to each other. The tumour volumes are calculated using the known formula $p \times L \times D^2/6$ (see Evans, B.D., *et al.*, Brit. J. Cancer 45, 466-468 (1982)). The results are given as treatment/control percentages (T/C x 100 = T/C %). At a dose of from 3 to 50 mg/kg active ingredient, distinct inhibition of the tumour growth is found, for example T/C % values of less than 10, which indicates strong inhibition of tumour growth.

The radicicol analogs for use in the invention can be used both alone and in combination with other pharmacologically active compounds, for example together with inhibitors of the enzymes of polyamine synthesis, inhibitors of protein kinase C, inhibitors of other tyrosine kinases, cytokines, negative growth regulators, for example TGF-ß or IFN-ß, aromatase inhibitors, antioestrogens and/or cytostatic agents.

Characteristically when the radicicol analogs are use to prevent or reverse multidrug resistance of tumour and other malignant cells, they are used in combination with cytostatic or cytotoxic agents. A suitable cell-based assay for assessing utility in restoring sensitivity of cancer cells to anti-neoplastic/cytotoxic, drug substances *in vitro* is as follows.

Cancer cell lines (CCL), e.g. from human small cell carcinoma of the lung, resistant to one or more cancer therapeutic drug substances (CTDS) selected from the group comprising Daunorubicin (DR); Vincristine (VC); Adriamycin (AM); Etoposide (ET); Tenoposide (TE); Colchicine (CC); and Taxol are developed in accordance with the methods described by Twentyman et al., Br. J. Cancer, 54, 253 (1986).

Sensitivity of resistant sub-lines (CCL-R) is compared with parental sensitive lines (CCL-S)by assaying inhibition of cell growth during continuous CTDS exposure, e.g. in the case of a DR-resistant line (CCL-DRR)by comparing growth of CCL-DRS and CCL-DRR lines in the presence of DR contained in the growth medium *ab initio*. For the purpose, cell proliferation is measured by cell counting using an electronic cell counter, counting being effected close to the termination of the exponential growth phase. CCL-R lines are selected for which the IC_{80} (drug concentration, e.g. DR concentration, required to reduce final cell number to 20% of that for non-CTDS (e.g. DR) treated controls is >80 X, preferably >100 X, greater than that of the parental CCL-S lines.

Sensitivity of selected CCL-R lines to CTDS (e.g. DR) in the presence or absence of test radicicol analog is then performed, employing cell counting as a measure of proliferation as

described above. For this purpose cells are cultured *ab initio* in the presence of varying concentrations of both CTDS and test radicicol analog. For screening, concentrations of the latter are chosen which do not themselves cause a significant reduction in proliferation. Appropriate concentrations are established by culturing CCL-S and CCL-R in the presence of varying concentrations of radicicol analog in the absence of CTDS. Radicicol analogs are routinely tested at concentrations of from 0.01 to 50, in particular 0.1 to 10, μg/ml, e.g. at concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 50 μg/ml. The ratio of CTDS (e.g. DR) required to inhibit cell proliferation by 50% in the absence of test radicicol analog (IC_{50}-CS) compared with that obtained in the presence of test radicicol analog (IC_{50}+CS) is taken as a measure of increased sensitivity of the CCL-R line to CTDS which has been induced by the radicicol analog. Stability of the CCL-R line used is ensured by cross checking its sensitivity to CTDS with that previously established.

Additional procedures for assessing utility in restoring sensitivity of cancer cells to anti-neoplastic/cytotoxic, drug substances, including *in vivo* procedures are described in EP 0296122 B, the relevant disclosures of which are incorporated by reference in the teaching of the present application.

Compounds for use in the Invention can be used both alone and in combination with other pharmacologically active compounds, for example in cancer treatment the compounds may be used together with inhibitors of the enzymes of polyamine synthesis, inhibitors of protein kinase C, inhibitors of other tyrosine kinases, cytokines, negative growth regulators, for example TGF-ß or IFN-ß, aromatase inhibitors, antioestrogens and/or cytostatic agents.

Suitable pharmaceutical compositions comprising Compounds for use in the invention as active ingredient and that can be used especially in the treatment of the diseases mentioned above include compositions for enteral, such as nasal, buccal, rectal or especially oral, administration and parenteral, such as intravenous, intramuscular or subcutaneous, administration to warm-blooded animals, especially human beings. The compositions comprise the active ingredient on its own or preferably together with a pharmaceutically acceptable carrier. The dosage of the

active ingredient depends on the disease to be treated, and on species, age, weight and individual condition, individual pharmacokinetic conditions, and the mode of administration.

The pharmaceutical compositions may comprise from approximately 1 % to approximately 95 % active ingredient, forms of administration in single dose form preferably comprising from approximately 20 % to approximately 90 % active ingredient and forms of administration that are not in single dose form preferably comprising from approximately 5 % to approximately 20 % active ingredient. Unit dose forms are, for example, dragées, tablets, ampoules, vials, suppositories or capsules. Other forms of administration are, for example, ointments, creams, pastes, foams, tinctures, lipsticks, drops, sprays, dispersions, etc. Examples are capsules comprising from approximately 0.05 g to approximately 1.0 g of the active ingredient.

The pharmaceutical compositions are prepared in a manner known *per se*, for example by means of conventional mixing, granulating, confectioning, dissolving or lyophilising procedures.

Solutions of the active ingredient, and also suspensions or dispersions, especially isotonic aqueous solutions, dispersions or suspensions, are preferably used, it being possible, for example in the case of lyophilised compositions that contain the active ingredient alone or together with a carrier, for example mannitol, for such solutions, suspensions or dispersions to be made up prior to use. The pharmaceutical compositions may be sterilised and/or may comprise excipients, for example preservatives, stabilisers, wetting agents and/or emulsifiers, solubilisers, salts for regulating the osmotic pressure and/or buffers, and are prepared in a manner known *per se*, for example by means of conventional dissolving or lyophilising procedures. The said solutions or suspensions may comprise viscosity-increasing substances, such as sodium carboxymethyl-cellulose, dextran, polyvinylpyrrolidone or gelatin.

Suspensions in oil comprise as the oil component the vegetable, synthetic or semi-synthetic oils customary for injection purposes. There may be mentioned as such especially liquid fatty acid esters that contain as acid component a long-chained fatty acid having from 8 to 22, especially from 12 to 22, carbon atoms, for example lauric acid, tridecylic acid, myristic acid,

pentadecylic acid, palmitic acid, margaric acid, stearic acid, arachidic acid, behenic acid, or corresponding unsaturated acids, for example oleic acid, elaidic acid, erucic acid, brassidic acid or linoleic acid, if desired with the addition of antioxidants, for example vitamin E, β-carotene or 3,5-di-tert-butyl-4-hydroxytoluene. The alcohol component of those fatty acid esters has a maximum of 6 carbon atoms and is a mono- or poly-hydric, for example a mono-, di- or tri--hydric, alcohol, for example methanol, ethanol, propanol, butanol or pentanol or the isomers thereof, but especially glycol and glycerol. The following examples of fatty acid esters are therefore to be mentioned: ethyl oleate, isopropyl myristate, isopropyl palmitate, "Labrafil M 2375" (polyoxyethylene glycerol trioleate, Gattefossé, Paris), "Labrafil M 1944 CS" (unsaturated polyglycolised glycerides prepared by alcoholysis of apricot kernel oil and consisting of glycerides and polyethylene glycol ester; Gattefossé, France), "Labrasol" (saturated polyglycolised glycerides prepared by alcoholysis of TCM and consisting of glycerides and polyethylene glycol ester; Gattefossé, France) and/or "Miglyol 812" (triglyceride of saturated fatty acids with a chain length of C_8 to C_{12}, Hüls AG, Germany), but especially vegetable oils, such as cottonseed oil, almond oil, olive oil, castor oil, sesame oil, soybean oil and more especially groundnut oil.

The injection compositions are prepared in customary manner under sterile conditions; the same applies also to introducing the compositions into, for example, ampoules or vials and to sealing the containers.

Pharmaceutical compositions for oral administration can be obtained, for example, by combining the active ingredient with one or more solid carriers, if desired granulating a resulting mixture, and processing the mixture or granules, if desired, and if necessary by the addition of additional excipients, to form tablets or dragée cores.

Suitable carriers are especially fillers, such as sugars, for example lactose, saccharose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, and also binders, such as starches, for example corn, wheat, rice or potato starch, methylcellulose, hydroxypropylmethylcellulose, sodium carboxy-

methylcellulose and/or polyvinylpyrrolidone, and/or, if desired, disintegrators, such as the above-mentioned starches, also carboxymethyl starch, crosslinked polyvinylpyrrolidone, or alginic acid or a salt thereof, such as sodium alginate. Additional excipients are especially flow conditioners and lubricants, for example silicic acid, talc, stearic acid or salts thereof, such as magnesium or calcium stearate, and/or polyethylene glycol, or derivatives thereof.

Dragée cores can be provided with suitable, optionally enteric, coatings, there being used *inter alia* concentrated sugar solutions which may contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, or coating solutions in suitable organic solvents or solvent mixtures, or, for the production of enteric coatings, solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethylcellulose phthalate. Colourings or pigments may be added to the tablets or dragée coatings, for example for identification purposes or to indicate different doses of active ingredient.

Orally administrable pharmaceutical compositions also include dry-filled capsules consisting of gelatin, and also soft, sealed capsules consisting of gelatin and a plasticiser, such as glycerol or sorbitol. The dry-filled capsules may contain the active ingredient in the form of granules, for example in admixture with fillers, such as corn starch, binders and/or glidants, such as talc or magnesium stearate, and optionally stabilisers. In soft capsules, the active ingredient is preferably dissolved or suspended in suitable liquid excipients, such as fatty oils, paraffin oil or liquid polyethylene glycols or fatty acid esters of ethylene or propylene glycol, to which stabilisers and detergents, for example of the polyoxyethylene sorbitan fatty acid ester type, may also be added.

Other oral forms of administration are, for example, syrups prepared in customary manner which comprise the active ingredient, for example, in suspended form and in a concentration of about 5 % to 20 %, preferably about 10 %, or in a similar concentration that provides a suitable single dose, for example, when administered in measures of 5 or 10 ml. Also suitable are, for example, powdered or liquid concentrates for the preparation of shakes, for example in milk. Such concentrates may also be packaged in single dose quantities.

Suitable rectally administrable pharmaceutical compositions are, for example, suppositories that consist of a combination of the active ingredient and a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols or higher alkanols.

For parenteral administration there are suitable especially aqueous solutions of an active ingredient in water-soluble form, for example in the form of a water-soluble salt, or aqueous injection suspensions that contain viscosity-increasing substances, for example sodium carboxymethylcellulose, sorbitol and/or dextran, and, if desired, stabilisers. The active ingredient, optionally together with excipients, can also be in the form of a lyophilisate and can be made into a solution prior to parenteral administration by the addition of suitable solvents.

The Compounds for use in the invention can be administered, prophylactically or therapeutically, as such or in the form of pharmaceutical compositions, preferably in an amount effective against the said diseases, to a warm-blooded animal, for example a human being, requiring such treatment, the compounds being used especially in the form of pharmaceutical compositions. In such treatment an individual of about 70 kg body weight will be administered a daily dose of from approximately 0.1 g to approximately 5 g, preferably from approximately 0.5 g to approximately 2 g, of a compound of formula II.

The following Examples serve to illustrate the invention and refer to the accompanying Figures, in which

Figure 1 which shows the 30 bp fragment used as a mRNA instability sequence in the reporter gene assay of Example 1;

Figure 2 which shows plasmid diagrams for pGL2_Neo30 and pGL2-Control; and

Figure 3 shows graphs of luciferase activity from clones 53 (solid bars) and 63 (open bars) treated with various concentrations of radicicol analog A (SDZ 216-732).

EXAMPLES

Example 1: Reporter Gene Assay for compounds which destabilise mRNA

A. Construction of pGL2_neo30

In order to obtain a vector for stable integration into THP-1 cells, a XhoI - SalI fragment of the neo resistant gene (expressing aminoglycoside 3' phosphotransferase) derived from pMCIneo (Stratagene) is subcloned into the SalI site of pGL2-Control (Promega). This resulting plasmid was called pGL2_Neo. A 30bp fragment (containing three tandem AUUUA motifs, based on the IL-Iβ 3'UTR sequence) obtained by annealing two complementary synthetic oligonucleotides (see Fig 1) is subcloned into pGL2_Neo using the PflM1 restriction site. This results in the luciferase expression vector pGL2_Neo30 (Fig. 2). Fig. 1 shows the IL-1β 3'UTR sequence containing three tandem AUUUA motifs used for ligation into the PflMI site of pGL2_Neo.

B. Transfection and selection of stable cell lines

The resulting vectors pGL2_Neo30 and pGL2_Neo are transfected into THP-1 cells by electroporation. 10^7 cells/ml in 1.3mM KH_2P0_4, 7.36m.M Na_2HP0_4, 2.44mM KCl, 124mm NaCl, 5mM glucose, 9.6μM $MgCl_2$ and 16μM $CaCl_2$ pH 7.2 are transfected with 20μg of DNA in a Bio-Rad Gene Pulser (250V, 690μF and indefinite resistance) using a 0.4cm cuvette. Cells are subsequently cultured in RPMI medium containing 10%FBS, 2mM L-Gln (L-glutamine), 50μM 2mercaptoethanol and 600μg/ml of G418 (geneticin). After transfection of pGL2_Neo30 and pGL2_Neo into THP-1 cells, stable cell lines are obtained by selection for G418 resistance and assayed for luciferase activity. One cell line of each transfection is chosen for further analysis; the pGL2_Neo30 cell line is referred to as clone No. 63 and the pGL2_Neo cell line as clone No. 53. No endogenous luciferase activity could be detected in normal THP-1 cells.

The tissue culture and luciferase activity measurements are carried out as described below.

C. Tissue culture:

The transfected human monocytic leukemia cell lines, clones No. 53 and 63 are grown in RPMI medium supplemented with 110 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Gln and 2 g/1 $NaHCO_3$. Heat-treated FBS (5%) is added before use. The cells are grown to a density of 5x 10^5/ml and induced to differentiate with 100 U/ml (final concentration) γIFN. Three hours later, 10 µl of LPS (5µg/ml final concentration) is added. This time point is designated time 0. Compounds are added at various times after LPS addition as indicated.

D. Luciferase activity measurement:

In order to adapt the system to the use of 96 well plates, cells are grown in Packard flat bottom white polystyrene microplates (Cat. No.6005180) in RPM1 medium lacking phenol red (AMIMED). Cells are plated at 5x10^4/well. After treatment of the cells, luciferase is measured using the Packard Luc Lite system (Cat. No.601691 1) according to the manufacturer's instructions in a final volume of 205µl. Briefly, to a cell suspension of 5 x 10^5 cells/ml, γIFN (1000U/ml Boehringer Mannheim No. 1050494) to a final concentration of 100 U/ml and 0.25% (v/v) Luc Lite Enhancer is added. After a 3 hour incubation LPS (50µg/ml SIGMA L-8274) is added to give 5µg/ml final concentration. The cells are then plated at 5x10^4/100µl/well into flat bottom white polystyrene microplates (Packard, Cat. No. 6005180) and incubated for 16 hours. 5 µl of compound solution or control vehicle is then added and the cells are further incubated as indicated. 100 µl of luciferase substrate solution is added and the plates are covered with TopSeal-A press-on adhesive sealing film (Packard Cat.No. 6005185) before measuring luminescence with a Packard Top Count Scintillation Counter at 22°C. The luciferase signal is stable for at least 90 min.

Example 2: Effect of the radicicol analog A.

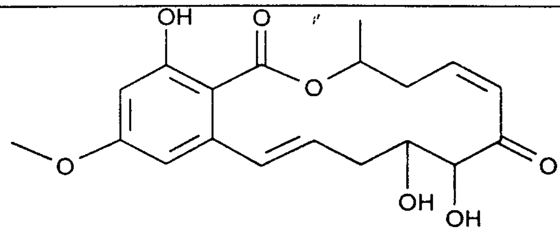
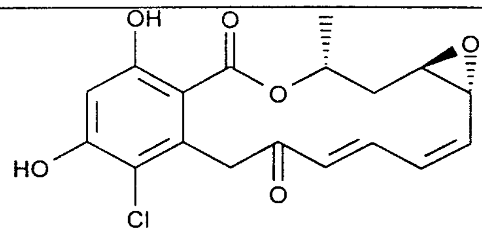
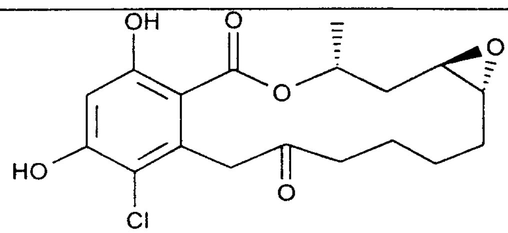
The THP-1 cell lines, clone No. 63 (containing PGL2_Neo30) and clone No. 53 (containing pGL2-Control) are grown, differentiated and stimulated with γIFN and LPS identical to normal THP-1 cells. Radicicol analog A is added 16 hours after the addition of LPS and cell extracts are then taken 8 hours later or as indicated. Luciferase activity is inhibited by 1 µM

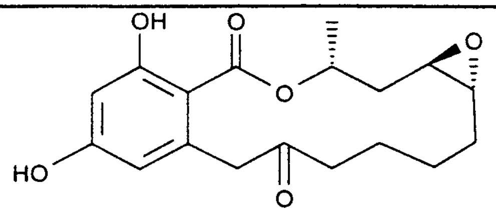
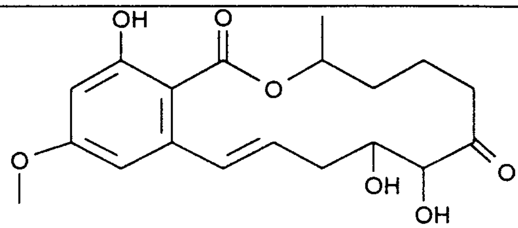
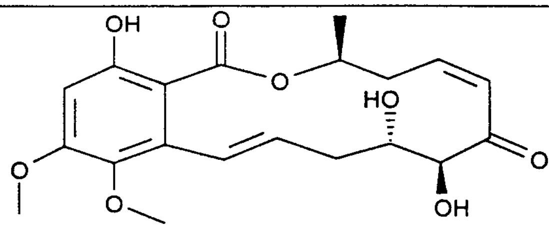
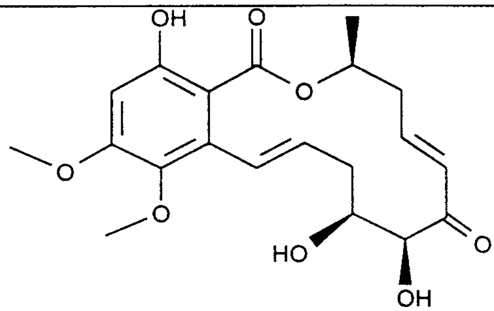
radicicol analog A on average by 50% +/-17%, in some cases inhibition was as great as 93%, whereas up to $5x10^{-6}M$ of radicicol analog A has no effects on the control clone No. 53, Fig. 3 (solid bars indicate clone No. 53, open bars clone No. 63).

Example 3: Application of Reporter Gene Assay to a number of radicicol analogs

A number of radicicol analogues are tested for their activity in the reporter gene assay substantially as described in the previous Examples. The results obtained are given in the Table below.

TABLE

COMPOUND	Luciferase reporter gene assay			
	clone	0.5µM	1µM	5µM
	53	114	105	107
	63	97	88	87
	53	68	51	40
	63	42	18	3
	53	99	77	69
	63	88	64	57

	53 83 81 70 63 80 66 61
	53 103 122 104 63 107 93 70
	53 136 140 108 63 69 32 9
	53 97 91 55 63 96 94 7

Example 4: Tablets, each comprising e.g. 50 mg of radicicol analog A or a pharmaceutically acceptable salt, are prepared as follows:

Composition (10000 tablets)

active ingredient	500.0 g
lactose	500.0 g
potato starch	352.0 g

gelatin	8.0 g
talc	60.0 g
magnesium stearate	10.0 g
silicon dioxide (highly dispersed)	20.0 g
ethanol	q.s.

The active ingredient is mixed with the lactose and 292 g of potato starch and the mixture is moistened with an ethanolic solution of the gelatin and granulated through a sieve. After drying, the remainder of the potato starch, the magnesium stearate, the talc and the silicon dioxide are mixed in and the mixture is compressed to form tablets, each weighing 145.0 mg and comprising 50.0 mg of active ingredient; the tablets may, if desired, be provided with breaking notches for finer adaptation of the dose.

Example 5: Film-coated tablet, each comprising 100 mg of radicicol analog A or a pharmaceutically acceptable salt are prepared as follows:

Composition (for 1000 film-coated tablets)	
active ingredient	100.0 g
lactose	100.0 g
corn starch	70.0 g
talc	60.0 g
calcium stearate	1.5 g
hydroxypropylmethylcellulose	2.36 g
shellac	0.64 g
water	q.s
methylene chloride	q.s.

The active ingredient, the lactose and 40 g of the corn starch are mixed and moistened with a paste prepared from 15 g of corn starch and water (with heating) and granulated. The granules

are dried, the remainder of the corn starch, the talcum and the calcium stearate are added and mixed with the granules. The mixture is compressed to form tablets (weight: 280 mg) which are then film-coated with a solution of the hydroxypropylmethylcellulose and the shellac in methylene chloride; final weight of the film-coated tablet: 283 mg.

Example 6: Hard gelatin capsules, comprising 100 mg of active ingredient, for example radicicol analog A or a pharmaceutically acceptable salt are prepared, for example, as follows:

Composition (for 1000 capsules)

active ingredient	100.0 g
lactose	250.0 g
microcrystalline cellulose	30.0 g
sodium lauryl sulfate	2.0 g
magnesium stearate	8.0 g

The sodium lauryl sulfate is added to the lyophilised active ingredient through a sieve of 0.2 mm mesh size. The two components are intimately mixed. Then first the lactose is added through a sieve of 0.6 mm mesh size and then the microcrystalline cellulose is added through a sieve of 0.9 mm mesh size. The mixture is then intimately mixed again for 10 minutes. Finally the magnesium stearate is added through a sieve of 0.8 mm mesh size. After mixing for a further 3 minutes, size 0 hard gelatin capsules are each filled with 390 mg of the resulting formulation. Soft gelatin capsules may be prepared using similar ingredients and procedures.

CLAIMS

1. A compound which induces degradation of mRNA which contains one or more mRNA instability sequences for use as a pharmaceutical, provided the compound is not radicicol analog A.

2. A method for the prophylaxis or treatment of a disease or medical condition having an etiology associated with the increased stability of mRNA which contains one or more mRNA instability sequences, comprising administering to a human or animal patient an effective amount of a compound which induces degradation of the mRNA, provided that the compound is not radicicol analog A when the disease or medical condition is one with an etiology associated with or comprising excessive cytokine release, particularly IL-1β release, such as rheumatoid arthritis, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma.

3. Use of a compound which induces degradation of mRNA which contains one or more mRNA instability sequences, for the preparation of a medicament for use in the treatment or prophylaxis of a disease or medical condition having an etiology associated with the increased stability of mRNA which contains one or more mRNA instability sequences, provided that the compound is not radicicol analog A when the disease or medical condition is one with an etiology associated with or comprising excessive cytokine release, particularly IL-1β release, such as rheumatoid arthritis, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma.

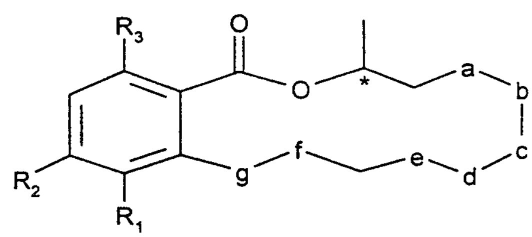
4. A method for inducing degradation of mRNA in a patient, which comprises administering an effective amount of a compound which induces mRNA degradation to the patient, wherein the mRNA contains an mRNA instability sequence, provided that the compound is not radicicol analog A when the mRNA is mRNA coding for IL-1β, IL–6 or TNF-α.

5. Use of a compound which induces mRNA degradation in the preparation of a medicament for use in inducing degradation of mRNA which contains a mRNA degradation sequence in a patient, provided that the compound is not radicicol analog A when the mRNA is mRNA coding for IL-1β, IL–6 or TNF-α.

6. Use of a radicicol analog for preparation of a medicament for treatment of a cancer and/or malignant disease.

7. A method for the prophylaxis or treatment of a cancer and/or malignant disease comprising administering to a patient an effective amount of a radicicol analog.

8. A use according to claims 3, 5 or 6 or method according to claim 2, 4 or 7 in which the compound or radicicol analog is a compound of formula II



II

wherein

R_1 is H, OH, halogen, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-;

R_2 is OH, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-;

R_3 is H, OH, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-;

-a-b- is -CHR_7-CHR_8- or cis or trans -CR_7=CR_8-,

wherein R_7 and R_8 are the same or different and are H, OH, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-, or

-a-b- is -CHR_7-CHR_8- and R_7 and R_8 together with O form an epoxide bridge;

c is >CH-OH, >C=O or >CH_2;

-d-e- is -CHR_7-CHR_8- or cis or trans -CR_7=CR_8-,

wherein R_7 and R_8 are the same or different and are H, OH, C_1-C_4 lower alkoxy, or C_1-C_4 lower alkyl-COO-, and

-f-g- is $-CH_2-CH_2-$, or cis or trans -CH=CH-,

or a pharmaceutically acceptable salt thereof or a physiologically-hydrolysable and -acceptable ester thereof.

9. A use according to claim 6 or 8 or a method according to claim 7 or 8 for treatment and prevention of oncogene mediated cancers and malignant diseases, to treat or prevent tumour growth and metastasis invasion in general, or to prevent or reverse multidrug resistance.

10. All novel compounds, methods and uses substantially as hereinbefore described with particular reference to the description and Examples.

```
 ATGGCTTCCCTATTTATTTATTTATTTGTTTGTCCAACCT
 ||||||||||||||||||||||||||||||||||||||
GGATACCGAAGGGATAAATAAATAAATAAACAAACAGGTT
```

FIGURE 1

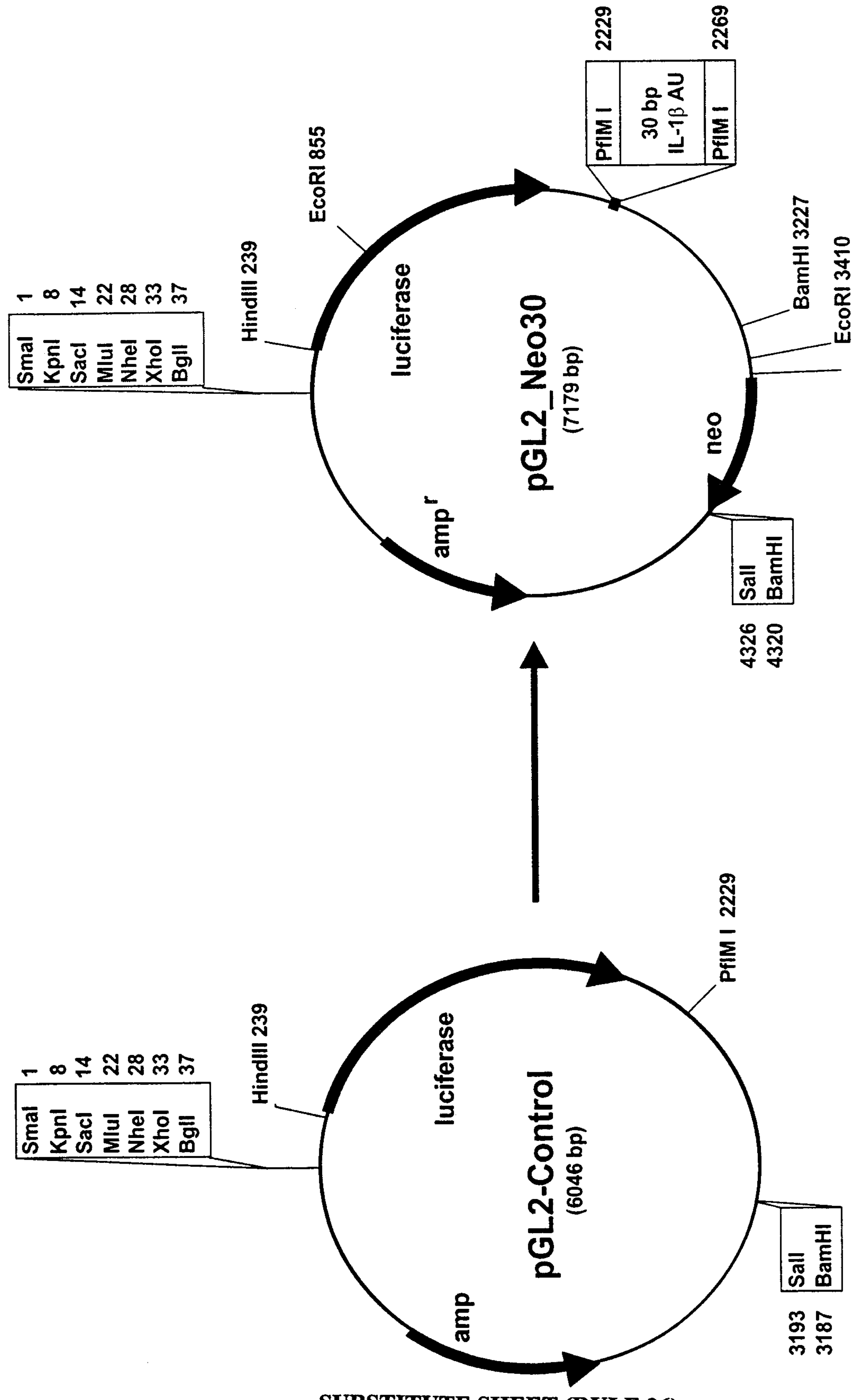

pGL2-Control
(6046 bp)
luciferase
amp
SmaI 1
KpnI 8
SacI 14
MluI 22
NheI 28
XhoI 33
BglI 37
HindIII 239
PflM I 2229
SalI 3193
BamHI 3187
pGL2_Neo30
(7179 bp)
luciferase
amp^r
neo
HindIII 239
EcoRI 855
PflM I
30 bp
IL-1β AU
PflM I
2229
2269
BamHI 3227
EcoRI 3410
MluI 3474
SalI 4326
BamHI 4320

FIGURE 2

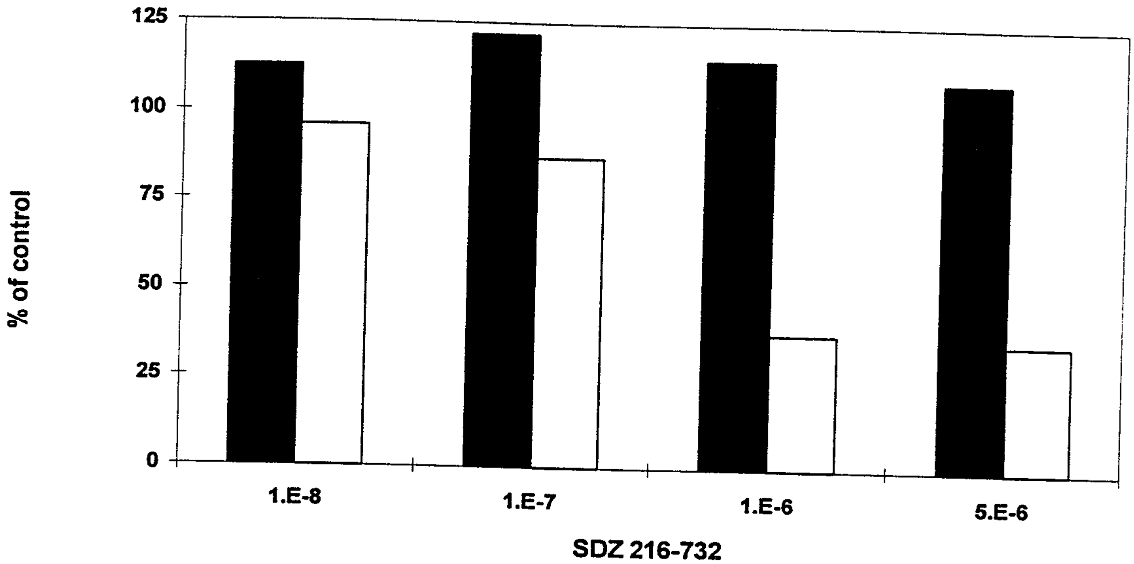


FIGURE 3

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/CA 99/01234

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/365 A61P31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 25928 A (SCRIPPS RESEARCH INST ;UNIV LOUISIANA STATE (US)) 29 August 1996 (1996-08-29) *cf. abstract, page 3, lines 5-15, page 5, 3rd para., page 10, last para. bridging with page 11, para. 1, page 14, 2nd para., page 17,lines 5-19, page 20, lines 7-23* ---	1-10

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	07/06/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Stoltner, A

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	FENG L., ET AL.: "Inhibition of protein tyrosine kinase, radicicol, suppresses the expression of cyclooxygenase and pro-inflammatory cytokines in LPS-stimulated rat alveolar macrophage in part by accelerating degradation of mRNA" ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, vol. 407, 1996, pages 281-288, XP000886787 *cf. introduction and page 283, paras. 2 and 4, page 284, last para. bridging with page 285, 1st para.*	1-10
Y	EP 0 606 044 A (SANDOZ LTD ;SANDOZ AG (DE); SANDOZ AG (AT)) 13 July 1994 (1994-07-13) cited in the application *cf. abstract, page 3, lines 50-53, page 8, lines 29-32*	1-10
Y	US 5 597 846 A (SUGIMURA YUKIO ET AL) 28 January 1997 (1997-01-28) *cf. abstract, col. 1, lines 30-40*	1-10
Y	EP 0 460 950 A (SANKYO CO) 11 December 1991 (1991-12-11) *cf. abstract and page 2, lines 12-18*	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No

PCT/CA 99/01234

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9625928 A	29-08-1996	US 5731343 A	24-03-1998
		AU 5175296 A	11-09-1996
		CA 2213632 A	29-08-1996
		EP 0810860 A	10-12-1997
EP 0606044 A	13-07-1994	AU 5211293 A	16-06-1994
		CA 2110553 A	05-06-1994
		CN 1095417 A	23-11-1994
		CZ 9302614 A	13-07-1994
		FI 935409 A	05-06-1994
		HU 65910 A	28-07-1994
		JP 6228122 A	16-08-1994
		MX 9307608 A	31-01-1995
		NO 934372 A	06-06-1994
		NZ 250344 A	26-10-1995
		PL 301281 A	13-06-1994
		SK 135493 A	10-08-1994
		ZA 9309088 A	05-06-1995
US 5597846 A	28-01-1997	AT 149498 T	15-03-1997
		CA 2044018 A	07-12-1991
		CN 1059720 A,B	25-03-1992
		CS 9101725 A	15-04-1992
		DE 69124844 D	10-04-1997
		EP 0460950 A	11-12-1991
		WO 9118905 A	12-12-1991
		HU 60743 A	28-10-1992
		JP 4226991 A	17-08-1992
		PT 97884 A,B	31-03-1992
		US 5650430 A	22-07-1997
EP 0460950 A	11-12-1991	AT 149498 T	15-03-1997
		CA 2044018 A	07-12-1991
		CN 1059720 A,B	25-03-1992
		CS 9101725 A	15-04-1992
		DE 69124844 D	10-04-1997
		WO 9118905 A	12-12-1991
		HU 60743 A	28-10-1992
		JP 4226991 A	17-08-1992
		PT 97884 A,B	31-03-1992
		US 5597846 A	28-01-1997
		US 5650430 A	22-07-1997

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Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/072844 A1

(54) Title: NOVEL EXPRESSION VECTORS

(57) Abstract: The present invention relates generally to expression vectors and their use in gene expression or gene regulation assays. More particularly, the present invention provides expression vectors and/or reporter vectors providing kinetics of protein expression with improved temporal correlation to promoter activity. Even more particularly, the invention provides expression vectors comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide. The present invention provides, *inter alia*, novel vectors, useful for identifying and analysing cis- and trans-acting regulatory sequences/factors as well as vectors and genetically modified cell lines or organisms that are particularly useful for drug screening and drug discovery.

NOVEL EXPRESSION VECTORS

FIELD OF THE INVENTION

The present invention relates generally to vectors and their use in gene expression or gene regulation assays. More particularly, the present invention provides expression vectors and/or reporter vectors providing kinetics of protein expression with improved temporal correlation to promoter activity. The present invention provides, *inter alia*, novel vectors and cell lines useful for modulating gene expression, identifying and analysing regulatory sequences, new targets and reagents for therapeutic intervention in human diseases and for drug-screening.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Reference herein to prior art, including any one or more prior art documents, is not to be taken as an acknowledgment, or suggestion, that said prior art is part of the state of the art.

The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating research and development in the medical and allied health fields. A particularly important area of research is the use of expression vectors to study gene expression. However, until now, a real-time analysis of gene expression has been limited by the lack of suitably designed vectors.

Reporter assays permit an understanding of what controls the expression of a gene of interest e.g., DNA sequences, transcription factors, RNA sequences, RNA-binding proteins, signal transduction pathways and specific stimuli.

Furthermore, reporter assays can be used to identify aspects of gene regulation that serve as new targets for therapeutic intervention in human disease. Reporter assays can potentially be used to screen drugs for their ability to modify gene expression. However, the cost and time

required for current reporter assay systems, together with the lengthy response times, has limited this application.

Genomic sequences have promoter sequences, generally upstream of the coding region, which dictate the cell specificity and inducibility of transcription and thereby affect the level of expression of protein products.

Specific sequence elements, typically rich in the nucleotide bases A and U and often located in the 3'-UTR of a gene, affect the stability of the mRNA and thereby affect the level of expression of the protein product. RNA-binding proteins bind certain mRNA sequences and thereby regulate mRNA stability and protein expression. Other sequences modulate translational efficiency.

A common application of gene reporter assays is the study of DNA sequences that regulate transcription. Typically, these sequences are located in the promoter region, 5' of the transcription start site. Such DNA elements are tested by cloning them into a similar site within a reporter plasmid, such that they drive and/or regulate transcription and therefore, expression of reporter protein. The reporter protein should be distinguishable from endogenous proteins and easily quantified. Various reporter proteins are used, the most common being luciferase, chloramphenicol transferase (CAT) and β galactosidase (β-gal).

The reporter protein is quantified in an appropriate assay and often expressed relative to the level of a control reporter driven by a ubiquitous promoter such as for example the promoter SV40. The control reporter must be distinguishable from the test reporter and is contained on a separate vector that is co-transfected with the test vector and used to control for transfection efficiency. Such assays are based on the premise that cells take up proportionally equal amounts of both vectors. Transient transfections of plasmid vectors are most commonly used.

The assays described above are used to identify a promoter region or the specific elements within a promoter. Alternatively, they are used to study the response to various stimuli of a

promoter or regulatory element. In some applications, the reporter constructs, or the transfected cells, are placed into an organism to study promoter function *in vivo*.

Another application of these reporter assays is the study or measurement of signal transduction pathways upstream of a specific promoter. For example, a promoter dependent on mitogen activated protein kinase (MAPK) for transcription can be linked to a reporter construct and used to measure the level of MAPK activation (or MAPK-dependent transcription) in cells. This technique can be utilized with a variety of informative promoters or enhancers and can be applied to cells or living organisms such as transgenic mice. For example, a photon camera can be used to measure luciferase reporter activity in whole mice containing a luciferase reporter linked to a promoter of interest (Contag, *et al*, 1997).

Luciferase is by far the most commonly used reporter assay for *in vitro* systems. The Dual Luciferase assay (DLA; Promega, Madison, WI, USA), is an improvement over other luciferase based systems in that both test and control reporter can essentially be measured in the same assay. As an example of current use, a typical DLA protocol is provided as follows:

The putative promoter element is cloned upstream of a firefly luciferase reporter gene such that it drives its expression. This plasmid is transiently transfected into a cell line, along with a control plasmid containing the *Renilla* luciferase gene driven by the SV40 promoter. ~2-50% of cells take up plasmid and express the reporters for ~3 days. The kinetics of expression involve an increase during the first ~24 h as luciferase protein accumulates, followed by a decrease from ~48 h as the number of plasmids maintained within the cells declines. 24-48 h after transfection, cells are harvested and lysed. Cell lysates are incubated with substrates specific to firefly luciferase and activity (light emission) is measured using a luminometer (96 well plate or individual samples). Additional substrates are then added, which inactivate firefly luciferase but allow *Renilla* luciferase to generate light. *Renilla* luciferase activity can then be measured.

The level of firefly luciferase activity is dependent, not only on promoter activity, but also on transfection efficiency. This varies greatly, depending on the amount of DNA, the quality of

the DNA preparation and the condition of the cells. The co-transfected control plasmid (*Renilla* luciferase driven by the SV40 promoter) is used to correct for these variables, based on the premise that *Renilla* luciferase activity is proportional to the amount of firefly luciferase plasmid taken up by the cells. Data are expressed as firefly luciferase activity / *Renilla* luciferase activity.

The disadvantages of the Dual Luciferase assay are as follows:

(i) Reagents are expensive and perishable and must be freshly prepared.

(ii) Generally this assay involves the preparation of cell lysates, which is time consuming and adds inaccuracy. e.g., loss of cells during lysis, pipetting errors, residual buffer/medium altering volumes.

(iii) Each sample yields only one datum point being the total activity of the cell population. No information is gained concerning the percentage of cells that express the reporter, nor the amount of expression per cell.

(iv) The transfection control (*Renilla*) does not always correct for huge variation in transfection efficiencies because:

a. Certain DNA preparations transfect/express poorly (perhaps due to reduced proportion of supercoiled DNA), but do not cause a corresponding decrease in the amount of co-transfected control plasmid.

b. There is evidence of cross-talk between the promoters of the two plasmids, such that control reporter activity is dependent on the construct with which it is co-transfected, e.g., expression of *Renilla* luciferase seems highest when co-transfected with a plasmid containing a strong promoter. Interference between promoters has also limited, if not prevented, the use of single plasmids expressing both test and control reporters.

c. A common application of both transcriptional and post-transcriptional studies is to measure activation/suppression by various stimuli (e.g., PMA, EGF, hormones). Unfortunately, SV40, RSV, TK and probably many other ubiquitously expressed promoters are activated by a variety of stimuli. Since these promoters are used to drive expression of the transfection control reporter (*Renilla*), these reporters do not give a true reflection of transfection efficiency following such treatments. (Ibrahim *et al.* 2000).

d. Differences in the half-lives of firefly vs *Renilla* luciferase proteins and perhaps mRNAs make the whole system very time-sensitive.

e. Rapidly diminishing light emission, particularly for *Renilla* luciferase, require absolute precision in the timing of measurement.

f. The relatively long half-lives of luciferase proteins and mRNAs, effectively mask temporal changes in transcription (e.g., following various stimuli or treatments).

In existing post-transcriptional/mRNA stability reporter assays, candidate elements, thought to affect mRNA stability are cloned into the corresponding region of a reporter vector (e.g., firefly luciferase) driven by a constitutive promoter such as SV40 or RSV. Changes in expression relative to the empty vector (same vector without element of interest) are assumed to be the result of altered mRNA stability. As with the preliminary described transfection assays, a transfection control plasmid (e.g., *Renilla* luciferase driven by a constitutive promoter such as SV40 or RSV) is co-transfected to allow correction for transfection efficiency. These assays suffer from the following additional disadvantages:

(i). Existing vectors were not designed for post-transcriptional studies and have no means for switching off transcription.

(ii). The purpose of these protocols is to study the post-transcriptional effects of candidate mRNA elements. However, these elements can also affect transcription of the reporter at the level of DNA. Furthermore, since the endogenous promoter of the gene of interest is not used, any transcriptional effects seen, may have little physiological relevance.

Systems for studying mRNA stability exist but involve direct measurement of the mRNA rather than a protein reporter. Due to the labor-intensive nature of protocols for quantifying mRNA, such systems are far more time consuming.

One system, for example, utilizes the *c-fos* promoter, which responds to serum induction with a brief burst of transcription. Putative instability elements are cloned into the 3-UTR of a Beta Globin (BBB) construct, which expresses a very stable mRNA under the control of a serum-inducible (*c-fos*) promoter. Transfected cells (generally NIH 3T3 cells) are first serum starved and then exposed to medium containing serum. The brief nature of the transcriptional response allows the kinetics of reporter mRNA degradation to be followed in a time course. These assays suffer from the following disadvantages:

(i). This assay is very time consuming and is therefore not applicable to rapid screening.

(ii). Can only be used in cells that support serum inducibility of the *c-fos* promoter. For example, many tumor cell lines maintain *c-fos* promoter activity in the absence of serum.

(iii). In cells such as NIH 3T3 cells, which do have the desired serum response, serum deprivation causes a cell cycle block and subsequent addition of serum, releases the cells from this block in a synchronous manner. Therefore, mRNA stability can only be measured in specific stages of the cell cycle.

(iv). In addition to activating the *c-fos* promoter, serum activates a multitude of other pathways, which introduce unwanted variables and prevent the study of more specific stimuli.

In another assay, cells are treated with drugs, such as Actinomycin D that inhibit transcription from all genes. The mRNA levels are measured in a time course to determine mRNA degradation rates. This system is used to study endogenous genes and suffer from the following disadvantages:

(i). Transcriptional inhibitors are extremely toxic at doses required such that mRNA stability is often being measured in stressed or dying cells.

(ii). Transcription inhibitors possess numerous unwanted activities including stabilization of certain mRNAs.

(iii). The process blocks transcription from all genes such that many signal transduction cascades are blocked, whereas others are activated. Therefore, results may not be physiologically relevant.

(iv). The technique is extremely labor intensive.

(v). The technique is highly variable within and between assays.

(vi). The technique is often not sensitive enough for transient transfection reporter assays, particularly in cells with a low transfection efficiency.

There is a need therefore to develop improved vectors and systems for conducting gene expression assays and in particular post-translational and post-transcriptional assays as well as assays that permit a more real-time determination of changes in gene expression.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

In accordance with the present invention, the inventor has developed a series of vectors and methods which permit *inter alia* modulation and determination of transcript stability and/or improved real-time determination of gene expression.

Nucleotide sequences are referred to by sequence identifier numbers (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1, <400>2, etc. A sequence listing is provided after the claims.

One aspect of the present invention is directed to an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide. As used herein the stability of a transcript may correspond to the half-life of the transcript.

Another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript.

In a related embodiment the present invention contemplates an expression vector comprising

a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a stabilising element which increases the stability of said transcript.

Another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element wherein said RNA element is a destabilising element which reduces the stability of a transcript corresponding to said transcribable polynucleotide and wherein said transcribable polynucleotide comprises a polynucleotide of interest and encodes a polypeptide.

Yet another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element wherein said RNA element is a destabilising element which reduces the stability of a transcript corresponding to said transcribable polynucleotide and wherein said transcribable polynucleotide comprises a polynucleotide of interest and encodes a reporter polypeptide.

Even yet another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element wherein said RNA element is a destabilising element which reduces the stability of said transcript and wherein said transcribable polynucleotide comprises a sequence of nucleotides encoding a reporter polypeptide.

Still another aspect of the present invention is directed to an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of a transcript corresponding to said transcribable polynucleotide and wherein said vector comprises one or more members selected from the group consisting of:

(i) a multiple cloning site for introducing a sequence of nucleotides;

(ii) a reporter gene;

(iii) a promoter and/or enhancer for regulating expression of said transcribable polynucleotide;

(iv) a polyadenylation sequence;

(v) a selectable marker gene; and

(vi) an origin of replication.

Even still another aspect of the present invention contemplates a cell containing a vector according to the present invention comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide.

Still another aspect of the present invention contemplates a cell containing a vector according to the presentinvention comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript.

A related aspect of the instant invention considers a genetically modified non-human organism comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript.

Yet a further embodiment of the present invention contemplates a method for determining expression of a polynucleotide of interest, said method comprising expressing said

polynucleotide of interest from an expression vector for a time and under conditions sufficient for RNA and protein synthesis to occur, said vector comprising a transcribable polynucleotide which comprises said polynucleotide of interest and a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; and wherein said expression vector comprises one or more members selected from the group consisting of:

(i) a multiple cloning site for introducing a sequence of nucleotides;

(ii) a reporter gene;

(iii) a promoter and/or enhancer for regulating expression of said transcribable polynucleotide;

(iv) a polyadenylation sequence;

(v) a selectable marker gene; and

(vi) an origin of replication;

and measuring the level and/or functional activity of an expression product of the transcribable polynucleotide over time compared to a control wherein said element enhances the temporal correlation between the activity of the promoter and/or enhancer that is operably connected to said transcribable polynucleotide and the level and/or functional activity of said expression product.

In still yet a further embodiment, the present invention contemplates a method for identifying a nucleotide sequence encoding an RNA element which modulates the stability of an RNA transcript, said method comprising introducing a test nucleotide sequence into an expression vector whereby said nucleotide sequence is connected to a polynucleotide encoding a reporter protein to form a transcribable polynucleotide which is operably connected to a promoter

and/or enhancer; expressing said transcribable polynucleotide for a time and under conditions sufficient for RNA and protein synthesis to occur; and wherein said expression vector comprises one or more members selected from the group consisting of:

(i) a multiple cloning site for introducing said test nucleotides sequence;

(ii) a polyadenylation sequence;

(iii) a selectable marker gene; and

(iv) an origin of replication;

and measuring the level and/or functional activity of an expression product of said transcribable polynucleotide over time compared to that of a control vector in the absence of said nucleotide sequence, wherein a level and/or functional activity which is different to that of the control vector over that time is indicative of a nucleotide sequence that encodes said RNA element.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of expression vectors encoding a destabilising mRNA.

Figure 2 is a schematic representation of transcription reporter vectors; Figure 2a shows vector series 2; Figure 2b shows vector series 3 and Figure 2c shows vector series 4.

Figure 3 is a schematic representation of Bi-directional transcription reporter vectors; Figure 3a shows vector series 5 and Figure 3b shows vector series 6.

Figure 4 is a schematic representation of reporter vectors for studying post-transcriptional regulation; Figure 4a shows vector series 7 and Figure 4b shows vector series 8.

Figure 5 is a graphical representation showing reporter activity as a measure against the amount of DNA transfected. A single DNA preparation of a plasmid encoding firefly luciferase was mixed at a 30:1 ratio with a separate plasmid encoding *Renilla* luciferase. Both DNA preparations appeared normal in spectrophotometry (OD260/280) and on ethidium bromide stained agarose gels (data not shown). Different volumes of this mixture were transfected into cells such that the total quantity of DNA was 1, 2 or 3 micrograms but the ratio of firefly to *Renilla* plasmids remained the same.

Fig. 5A is a graphical representation showing that *Renilla* luciferase activity was dependent on the amount of DNA transfected. However, firefly luciferase activity (Fig. 5B) did not increase with increasing amounts of DNA, perhaps because the firefly DNA preparation was of poor quality. Consequently, the firefly/*Renilla* ratio (Fig. 5C), which would typically be used as a measure of the firefly promoter activity, varied considerably depending on the amount of DNA used. These data demonstrate that co-transfections with *Renilla* plasmids do not adequately control for the transfection efficiency of the firefly plasmid

Figure 6 is a graphical representation showing reporter activity for various promoter systems using the Dual Luciferase Assay. Six different promoter fragments (numbered 1-6) were cloned

into pGL3 firefly luciferase plasmids. One microgram of each clone was co-transfected with 30ng of *Renilla* (transfection control) plasmid, driven by an SV40 promoter. Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Assay (Promega, Madison, WI, USA). Results are expressed as *Renilla* luciferase activity (A), Firefly luciferase activity (B) and firefly divided by *Renilla* activity (C). Similar results were seen in multiple experiments using at least 2 different preparations of each construct.

Renilla luciferase activity (Fig. 6A) is intended as a transfection control and analysis of this result alone would suggest an unusually high variation in transfection efficiency. For example, *Renilla* luciferase activity is 3.5 fold higher when co-transfected with construct 4 compared to co-transfection with construct 3. Variations in DNA quality or errors in the quantification of DNA seem unlikely as sources of error since the same pattern was seen with a separate set of DNA preparations (data not shown).

Firefly luciferase activity (Fig. 6B) is influenced by both transfection efficiency and differences between promoters 1-6. The pattern of differences is similar to that seen with *Renilla* (Fig. 6A). For example, 3 and 6 are low whilst 4 and 5 are high. However these differences between constructs are more marked with firefly (e.g., construct 4 is 12 fold higher than construct 3), suggesting that the activity of promoters 1-6 is somehow affecting expression of *Renilla* (or vice versa).

Firefly/*Renilla* (Fig. 6C) is considered to be a measure of true firefly promoter activity (1-6) after correction for transfection efficiency (*Renilla*). Again a similar pattern is seen, suggesting that indeed 3 and 6 are the weakest promoters whilst 4 and 5 are the strongest. Whilst it is possible that promoter activity (Fig. 6C) coincidentally correlated with transfection efficiency (Fig. 6A), this possibility seems extremely unlikely given that similar results were obtained with numerous different constructs and multiple different preparations of the same construct. It seems more likely that the level of expression of *Renilla* luciferase is affected by the strength of the promoter construct with which it is co-transfected. Consequently, apparent differences between promoters 1-6 are likely to be an underestimation of the true differences.

Figure 7 is a graphical representation showing different reporter levels for BTL, BTG2, BTG1 and BTG1N4 expression vectors on a time course after blocking transcription. Tet-Off HeLa cells were transfected with the following reporter plasmids, each containing a TRE promoter linked to a reporter gene; BTL (luciferase), BTG2 (d2EGFP), BTG1 (d1EGFP) and BTG1N4 (same as BTG1 but with 4 copies of the nonamer UUAUUUAUU present in the 3'UTR-encoding region). Ten hrs after transfection, each flask of cells was split into multiple small plates. Doxycycline (1□g/ml) was added at 24 hrs after transfection (time zero) to block transcription of the reporter genes. Reporter levels (fluorescence or luminescence) were measured at this and subsequent time points, as described in Exmaple 14, and presented as the percentage of time zero. No decrease in luciferase activity (BTL) was seen during the 10 hr time-course. The 2 hr half-life EGFP construct (BTG2) showed a moderate response to the doxycycline-induced block in transcription and a faster response was seen with the 1 hr half-life EGFP (BTG1). The construct containing the nonamers (BTG1N4), however, showed by far the fastest response to this block in transcription.

Figure 8 is a graphical representation showing the data used for Fig. 7 displayed on a linear scale. The doxycycline-induced block in transcription is detectable as a 50% block in reporter levels after approximately 6.5 hrs with BTG1. However, this is reduced to less than 3 hrs by inclusion of the nonamers (BTG1N4).

Figure 9 is a graphical representation showing the effect of different numbers (1, 2 or 4) of nonamer RNA destabilising elements. A time-course was performed as described in Fig. 7, except with time zero defined as 4 hrs after addition of doxycycline to eliminate the effect of the delay in the action of this drug. The presence of a single nonamer (BTG1N1) was sufficient to increase the "effective rate of decay," whereas progressively stronger effects were seen with 2 nonamers (BTG1N2) and 4 nonamers (BTG1N4). The latter construct showed an "effective half-life" of ~1 hr 20 mins, which is little more than the 1 hr half-life of the protein alone.

Figure 10 is a graphical representation showing changing reporter levels over time in the absence of a transcriptional block. A time-course was performed as described in Fig. 7. However, the data presented represent samples not treated with doxycycline and measured at

24 hrs after transfection (start) or 34 hrs after transfection (finish). Consistent expression levels were seen only with BTG1N4.

Figure 11 is a graphical representation showing changes in reporter levels over time in the absence of a transcriptional block. A time-course was performed as described in Fig. 7. BTG1fos contains the c-fos ARE. These data demonstrate that different types of mRNA destabilising elements can be used to achieve the same effect.

Figure 12 is a graphical representation showing that RNA destabilising elements are useful in determining expression when a Luciferase reporter protein is used. A further enhancement would be expected using a luciferase reporter protein with protein destabilising elements. A time-course was performed as described in Fig. 7, using two luciferase-expressing constructs. BTL contains the standard Firefly luciferase-coding region and 3'UTR (derived from pGL3-Basic; Promega), whereas BTLN6 contains 6 copies of the nonamer UUAUUUAUU in the 3'UTR.

Figure 13 is a graphical representation showing reporter levels over time using DsRed destabilised by RNA destabilising elements and protein destabilising elements. A time course was performed as described in Fig. 7 and Example 14. The constructs used were DsRed2 (BTR), DsRed-MODC (BTR1) and DsRed-MODC containing 4 UUAUUUAUU nonamers in the 3'UTR (BTR1N4). After blocking transcription with doxycycline, red fluorescence continues to increase with all constructs. This is substantially reduced by the protein destabilising element and further reduced by the mRNA destabilising element.

Figure 14 is a graphical representation showing a time-course was performed as described in Fig. 7. All of the mRNA destabilising elements tested were very effective at increasing the rate of decay compared to controls (BTG1). These data show that the c-myc ARE is an effective destabilising element (BTG1myc) and that a modest increase in destabilising activity can be obtained by combining the myc ARE with 4 nonamers (BTG1N4myc). Six nonamers (BTG1N6) also appeared to destabilise somewhat more than 4 nonamers (BTG1N4).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

By "*3'UTR*" is meant	the region of a polynucleotide downstream of the termination codon of a protein-encoding region of that polynucleotide, which is not translated to produce protein.
By "*5'UTR*" is meant	5' (upstream) untranslated region of an mRNA. Also used to refer to the DNA region encoding the 5'UTR of the mRNA.
By "*About*" is meant	a quantity, level, value, dimension, size, or amount that varies by as much as 30%, preferably by as much as 20%, and more preferably by as much as 10% to a reference quantity, level, value, dimension, size, or amount.
By "ARE" is meant	an AU-rich element in mRNA i.e., a sequence that contains a high proportion of adenine and uracil nucleotides. Also used to refer to the DNA region encoding such an mRNA element.
By "*Biologically active fragment*" is meant	a fragment of a full-length reference polynucleotide or polypeptide which fragment retains the activity of the reference polynucleotide or polypeptide, respectively.
By "*c-fos*" is meant	an immediate early gene, briefly induced by mitogenic signals
By "*CAT*:" is meant	Chloramphenicol acetyltransferase. A bacterial enzyme often used as a reporter.
By "*d1EGFP*" is meant	a variant of EGFP that is fused to a mutated PEST sequence and consequently has a half-life of only about 1 hour. Similarly, d1ECFP and d1EYFP are also available. A destabilised variant of DsRed could be made in the same way. Henceforth referred to as d1DsRed.

By "*d2EGFP*" is meant	a mutant form of EGFP variants that is fused to a PEST sequence and consequently has a half-life of only 2 hours. Similarly, d2ECFP (cyan) and d2EYFP (yellow) are also available. A destabilised variant of DsRed could possibly be made in the same way. Henceforth referred to as d2DsRed.
By "*dEGFP*" is meant	a general term for all destabilised variants of EGFP (including all colors) formed. (Li *et al*).
By "*DNA*" is meant	deoxyribonucleic acid.
By "*Derivative*" is meant	a polynucleotide or polypeptide that has been derived from a reference polynucleotide or polypeptide, respectively , for example by conjugation or complexing with other chemical moieties or by post-transcriptional or post-translational modification techniques as would be understood in the art.
By "*DsRed*" is meant	the red fluorescent protein isolated from the IndoPacific sea anemone relative *Discosoma* species.
By "*ECFP*" is meant	the mutant form of EGFP with altered excitation/emission spectra that fluoresces cyan colored light.
By "*EGF*" is meant	Epidermal growth factor
By "*EGFP*" is meant	the enhanced green fluorescent protein. A mutant form of GFP with enhanced fluorescence. (Cormack *et al*).
By "*ELISA*" is meant	the enzyme-linked immunosorbent assay
By "*ErbB2*" is meant	the second member of the epidermal growth factor receptor family. Also known as HER-2
By "*Exon*" is meant	the sequences of an RNA primary transcript that are part of a messenger RNA molecule, or the DNA that encodes such sequences. In the primary transcript neighbouring exons are separated by introns.
By "*Expression Vector*" is meant	a vector that allows a cloned segment of DNA to be expressed inside a cell.
By "*EYFP*" is meant	a mutant form of EGFP with altered excitation/emission spectra that fluoresces yellow colored light.

By "*Firefly Luciferase*" is meant the enzyme derived from the luc gene, cloned from the firefly. Catalyzes a reaction using D-luciferin and ATP in the presence of oxygen and Mg^{++} resulting in light emission. Often used as a reporter.

By "*Flow Cytometry*" is meant a method, in which live or fixed cell suspensions are applied to a flow cytometer that individually measures an activity or property of a detectable label associated with the cells of the suspension. Labelling of cells can occur, for example, via fluorescent compounds or by antibodies covalently attached to a specific fluorescent compound. Several different excitation/emission wavelengths can be tested simultaneously to measure different types of fluorescence. Sub-populations of cells with desired characteristics (fluorescence, cell size) can be gated such that further statistical analyses apply only to the gated cells. Flow cytometers equipped with a cell sorting option can physically separate cells with the desired fluorescence and retrieve those (live) cells in a tube separate from the remainder of the initial cell population. Also referred to as FACS (fluorescence activated cell sorting).

By "*Gene*" is meant the segment of DNA that encodes a RNA molecule. The term “gene” sometimes but not always includes the promoter region.

By "*GFP*" is meant a fluorescent protein (Tsien *et al*), which is isolatable from the jellyfish *Aequoria victoria*, and which can be used as a reporter protein. DNA constructs encoding GFP can be expressed in mammalian cells and cause the cells to fluoresce green light when excited with specific wavelengths. The term “GFP” is used herein to refer to all homologues and analogues, including colour variants and fluorescent proteins derived from organisms other than *Aequoria victoria* (e.g., DSRed, Clonetech; hrGFP, Stratagene).

By "*Half-life*" is meant the time taken for half of the activity, amount or number of molecules to be eliminated.

By "*Intron*" is meant a non-coding sequence within a gene, or its primary transcript, that is removed from the primary transcript and is not present in a corresponding messenger RNA molecule.

By "*Luciferase*" is meant the commonly used reporter enzyme that catalyses a reaction, which leads to light emission. Exogenous substrates are added and the reaction is quantified using a luminometer. The substrate requirements for firefly and *Renilla* luciferases are different, allowing the two to be distinguished in the Dual Luciferase Assay (Promega, Madison, WI, USA).

By "*MAPK*" is meant Mitogen Activated Protein Kinase. Includes several different kinases involved in intracellular signal transduction pathways that lead to growth or apoptosis (cell death). The term "MAPK" is sometimes used in reference to two specific MAPKs, Erk1 and Erk2 (extracellular regulated kinases 1 and 2).

By "*MCMV*" is meant Minimal CMV promoter. Does not activate transcription on its own but can be linked to a TRE to provide tetracycline (and doxycycline)-dependent transcription.

By "*MCS*" is meant Multiple Cloning Site. The region of a DNA vector that contains unique restriction enzyme recognition sites into which a DNA fragment can be inserted. The term "MCS" as used herein, also includes any other site that assists the insertion of DNA fragments into the vector. For example, a T overhang (Promega, Madison, WI, USA), which allows direct insertion of fragments generated by polymerase chain reaction (PCR).

By "*mRNA*" is meant Messenger RNA. A "transcript" produced in a cell using DNA as a template, which itself encodes a protein. mRNA is typically comprised of a 5'UTR, a protein encoding region and a 3'UTR. mRNA has a limited half-life in cells, which is determined, in part, by stability elements, particularly within the 3'UTR but also in the 5'UTR and protein encoding region.

By "*MODC*" is meant Mouse ornithine decarboxylase or the portion and/or derivative thereof containing a PEST sequence.

By "*Modulating*" is meant increasing or decreasing, either directly or indirectly, the stability of a molecule of interest.

By "*operably connected*" or "*operably linked*" and the like is meant a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the nucleic acid sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences are ultimately processed to produce the desired protein. "Operably connecting" a promoter to a transcribable polynucleotide is meant placing the transcribable polynucleotide (e.g., protein encoding polynucleotide or other transcript) under the regulatory control of a promoter, which then controls the transcription and optionally translation of that polynucleotide. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position a promoter or variant thereof at a distance from the transcription start site of the transcribable polynucleotide, which is approximately the same as the distance between that promoter and the gene it controls in its natural setting; i.e.: the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of function. Similarly, the preferred positioning of a regulatory sequence element (e.g., an operator, enhancer etc) with respect to a transcribable polynucleotide to be placed under its control is defined by the positioning of the element in its natural setting; *i.e.* the genes from which it is derived.

The term "*pA*" is meant used in diagrams here to indicate a poly adenylation site. A DNA sequence that serves as the site to stop transcription and add to the immature mRNA, a polyA tail. Various pA sequences from SV40 virus genes or the β galactosidase gene or other sources, including synthetic polyadenylation sites can be used in expression vectors for this purpose.

The term "*PEST*" refers to an amino acid sequence that is enriched with the amino acids proline (P), glutamic acid (E), serine (S) and threonine (T). Proteins containing PEST sequences have shortened half-lives.

By "*Plasmid*" is meant a Circular DNA vector. Plasmids contain an origin of replication that allows many copies of the plasmid to be produced in a bacterial (or sometimes eukaryotic) cell without integration of the plasmid into the host cell DNA.

By "*Polynucleotide*" or "*Nucleic acid*" is meant linear sequences of nucleotides, including DNA or RNA, which may be double-stranded or single-stranded..

By "*Polypeptide*", "*Peptide*" or "*Protein*" is meant a polymer of amino acids joined by peptide bonds in a specific sequence.

By "*Promoter*" is meant a region of DNA, generally upstream (5') of the mRNA encoding region, which controls the initiation and level of transcription. This term also includes within its scope inducible, repressible and constitutive promoters.

By "*PMA*" is meant Phorbol myristoloic acid

By "*Renilla Luciferase*" is meant that derived from sea pansy (*Renilla reniformis*), utilizes oxygen and coelenterate luciferin (coelenterazine) to generate light emission

By "*Reporter Vector*" is meant a expression vector containing a "Reporter Gene" that encodes a protein or polypeptide (or mRNA) that can be easily assayed. Typically, the reporter gene is linked to regulatory sequences, the function or activity of which, is being tested.

By "*Reporter*" is meant	a molecule, typically a protein or polypeptide, that is encoded by a reporter gene and measured in a reporter assay. Current systems generally utilize an enzymatic reporter and measure reporter activity.
By "*RNA*" is meant	Ribonucleic Acid.
By "*rtTA*" is meant	Reverse tTA (see below), which binds the TRE and activates transcription only in the presence of tetracycline or doxycycline.
By "SEAP" is meant	Secreted alkaline phosphatase reporter gene.
By "*SKBR3*" is meant	the human breast cancer cell line that overexpresses ErbB2.
By "*Stringent conditions*" is meant	temperature and ionic conditions under which only nucleotide sequences having a high frequency of complementary bases will hybridise. The stringency required is nucleotide sequence dependent and depends upon the various components present during hybridisation and subsequent washes, and the time allowed for these processes. Generally, in order to maximise the hybridisation rate, non-stringent hybridisation conditions are selected; about 20 to 25° C lower than the thermal melting point (T_m). The T_m is the temperature at which 50% of specific target sequence hybridises to a perfectly complementary probe in solution at a defined ionic strength and pH. Generally, in order to require at least about 85% nucleotide complementarity of hybridised sequences, highly stringent washing conditions are selected to be about 5 to 15° C lower than the T_m. In order to require at least about 70% nucleotide complementarity of hybridised sequences, moderately stringent washing conditions are selected to be about 15 to 30° C lower than the T_m. Highly permissive (low stringency) washing conditions may be as low as 50° C below the T_m, allowing a high level of mis-matching between hybridised sequences. Those skilled in the art will recognise that other physical and chemical parameters in the hybridisation and wash stages can also be altered to affect the outcome of a detectable hybridisation signal from a specific level of homology between target and probe sequences.

"*SV40/CMV/RSV*"	to refer to promoter elements derived from simian virus, cytomegalovirus and rous sarcoma virus respectively. Generally, these promoters are thought to be constitutively active in mammalian cells.
By "*TetO*" is meant	the Tet operator DNA sequence derived from the *E. coli* tetracycline-resistance operon.
By "*Tet-Off Cell Lines*" is meant	cell lines stably expressing tTA such that tetracycline or doxycycline will shut off transcription from TRE promoters.
By "*Tet-On Cell Lines*" is meant	cell lines stably expressing rtTA such that tetracycline or doxycycline will turn on transcription from TRE promoters.
By "*Transcription*" is meant	the process of synthesizing a RNA molecule complementary to the DNA template.
By "*Transfection*" is meant	the process during which a plasmid or DNA fragment is inserted into a eukaryotic cell. Typically, 2-50% of cells take up the plasmid and express the protein product for ~3 days without incorporating the plasmid DNA into the cell's chromosomes (= transient transfection). A small proportion of these cells will eventually incorporate the plasmid DNA into their gemone and permanently express the protein product (= stable transfection).
By "*Translation*" is meant	the process whereby an mRNA molecule is used as a template for protein synthesis.
By "*TRE*" is meant	here to define any Tetracycline Responsive Element (Gossen *et al*), generally combined with a minimal promoter such that transcription occurs only via the binding of exogenous factors (e.g., tTA or rtTA) to the TRE. Preferred embodiments of this invention utilize a TRE comprised of 7 repeats of the tetO sequence linked to a minimal CMV promoter (mCMV) (Clontech Laboratories Inc., Palo Alto, CA, USA).
By "t*TA*" is meant	Tetracycline-controlled transactivator, which is comprised of the Tet repressor protein (TetR) and the VP16 activation domain, such that it binds the TRE and activates transcription, only in the absence of tetracycline or doxycycline.

By "*TS*" is meant	Thromboxane synthase promoter.
By "*Variant*" is meant	a polynucleotide or polypeptide displaying substantial sequence identity with a reference polynucleotide or polypeptide, respectively. Variant polynucleotides also include polynucleotides that hybridise with a reference sequence under stringent conditions. These terms also encompasses polynucleotides which differ from a reference polynucleotide by the addition, deletion or substitution of at least one nucleotide. In this regard, it is well understood in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference polynucleotide whereby the altered polynucleotide retains the biological function or activity of the reference polynucleotide. The terms *"polynucleotide variant"* and *"variant"* also include naturally occurring allelic variants. With regard to variant polypeptides, it is well understood in the art for example that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the polypeptide (conservative substitutions).
By "*Vector*" is meant	a vehicle for inserting a foreign DNA sequence into a host cell and/or amplifying the DNA sequence in cells that support replication of the vector. Most commonly a plasmid but can also be a phagemid, bacteriophage, adenovirus or retrovirus.
By "vEGFP" "EGFP variants" or "variant of EGFP" is meant	the different color variants of EGFP and/or the different half-life variants.
By "vGFP" is meant	all variants of GFP; including homologues and analogues such as DsRed, also EGFP variants or destabilised GFP variants.

The present invention provides *inter alia* expression vectors which modulate the stability of transcripts and consequently, the amount of protein produced by the vector. Although expression vectors which increase the stability of a transcript are clearly encompassed by the present invention, a particularly preferred embodiment focuses on destabilising transcripts. Here transcript stability can be reduced by the addition of one or more destabilising elements to, or by the removal of one or more stability elements (e.g., a poly A tail) from, a transcribable polynucleotide. Compared to existing expression vectors, the vector of the present invention

provides kinetics of protein expression with improved temporal correlation to the promoter activity, e.g., by reducing the time lag between decreased promoter activity and decreased levels of a corresponding expression product.

Accordingly, one aspect of the present invention is directed to an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide.

The term “modulates” in the context of transcript stability refers to increasing or decreasing the stability of a transcript and optimal amounts of modulation depends upon the particular application. Without limiting the present invention to any one particular theory or mode of operation, where the RNA element is a sequence of nucleotides which destablilises the transcript, it is envisaged that the element directly or indirectly targets the transcript for degradation.

As used herein the term "destabilising element" refers to a sequence of amino acids or nucleotides which reduces the half-life of a protein or transcript, respectively, inside a cell. Accordingly, an "RNA destabilising element" comprises a sequence of nucleotides which reduces the intracellular half-life of an RNA transcript and a "protein destabilising element" comprises a sequence of amino acids which reduces the intracellular half-life of a protein.

The extent of the reduction sought depends upon the particular application. In a preferred embodiment the extent of RNA destabilisation significantly improves the temporal correlation between promoter activity and reporter levels or activity in expression vectors compared to vectors without destabilisation elements. In relation to increasing transcript stability, optimum levels of stability will again depend upon the application.

An "RNA stabilising element" is a sequence of nucleotides which increases the intracellular half-life.

"RNA" molecules include all RNA molecules such as mRNA, heterogenous nuclear RNA (hnRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small cytoplasmic RNA (scRNA), ribosomal RNA (rRNA), translational control RNA (tcRNA), transfer RNA (tRNA), eRNA, messenger-RNA-interfering complementary RNA (micRNA) or interference RNA (iRNA) and mitochondrial RNA (mtRNA).

Messenger RNA (mRNA) is a preferred form of RNA.

In the context of reducing the intracellular half-life of a molecule selected from an RNA transcript or an encoded protein of interest, (a) one or more destabilising elements and/or (b) one or more stabilising elements are typically chosen to confer a level of enhanced degradation on the molecule, which thereby reduce(s) the intracellular half-life of the molecule to a half-life that is suitably less than about 24 hours, more preferably less than about 10 hours, even more preferably less than about 5 hours, even more preferably less than about 3 hours, even more preferably less than about 1 hour, even more preferably less than about 30 minutes, even more preferably less than about 15 minutes, even more preferably less than about 10 minutes, even more preferably less than about 5 minutes, and still even more preferably less than about 3 minutes. The half-life of an RNA transcript or an encoded protein of interest preferably corresponds to the lowest half-life that provides a steady-state expression level of at least 10 fold the minimum detectable level of the transcript or encoded protein.

The intracellular or intracellular-like conditions are preferably physiological for the cell type. The temperature of the intracellular or intracellular-like conditions is preferably physiological for the cell type. Exemplary temperatures for mammalian cells range suitably from about 30° C to about 42° C, and preferably from about 35° C to about 37° C.

At a minimum, enhanced ribonucleic or proteolytic degradation of an RNA transcript or polypeptide, respectively, refers to a level of ribonucleic or proteolytic degradation that is at least about 5%, preferably at least about 10%, more preferably at least about 20%, even more preferably at least about 40%, even more preferably at least about 50%, even more preferably at least about 60%, even more preferably at least about 70%, even more preferably at least

about 80%, even more preferably at least about 90%, even more preferably at least about 100%, even more preferably at least about 150%, even more preferably at least about 200%, even more preferably at least about 400%, even more preferably at least about 600%, even more preferably at least about 1,000%, even more preferably at least about 2,000%, even more preferably at least about 4,000%, even more preferably at least about 6,000%, even more preferably at least about 8,000%, preferably at least about 10,000%, still even more preferably at least about 12,000%, greater than that of the RNA transcript or polypeptide in the absence of the destabilising element(s) or in the presence of a stabilising element(s). Assays for measuring RNA degradation are known to those of skill in the art. For example, RNA degradation can be measured using a range of assays disclosed for example by Ross, J (1995) or by Liu, J *et al.* (JBC 2000), which are based on the use of transcriptional inhibitors (Actinomycin D, DRB, cordycepin, alpha-amanitin), pulse labelling (radioactive nucleosides), cell-free decay methods (polysomes, cytosol or reticulocytes), or short-term promoter activation (fos promoter, see below). Assays for measuring degradation of proteins are also known to persons of skill in the art. For example, proteolytic degradation may be measured *in vitro* using a mammalian cell lysate assay including, but not restricted to, the reticulocyte lysate assay of Bachmair *et al* in U.S. Patent Serial No. 5,646,017. Alternatively, proteolytic degradation may be measured *in vivo* using cycloheximide or pulse-chase protocols as for example disclosed by Vazhappilly, R and Sucher, N (2002) or by Saito, T *et al.* (1998).

The RNA destabilising elements can be derived from any source and in particular the 3' UTR or 5' UTR regions of short-lived mRNAs often contain destabilising sequences. As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

The RNA destabilising sequences may be cloned from short-lived RNAs such as, for example; *c-fos, c-jun, c-myc, GM-CSF, IL-3, TNF-alpha, IL-2, IL-6, IL-8, IL-10, Urokinase, bcl-2, SGLT1 (Na(+)-coupled glucose transporter), Cox-2 (cyclooxygenase 2), IL8, PAI-2 (plasminogen activator inhibitor type 2), beta1-adrenergic receptor, GAP43* (5'UTR and 3'UTR).

AU-rich elements (AREs) and/or U-rich elements (UREs), including but not limited to single, tandem or multiple or overlapping copies of the nonamer UUAUUUA(U/A)(U/A) (where U/A is either an A or a U) (Lagnado *et al* 1994) and/or the pentamer AUUUA (Xu *et al* 997) and/or the tetramer AUUU (Zubiaga *et al.* 1995).

RNA destabilising elements have also been described for example from phosphoenolpyruvate carboxy kinase mRNA (PEPCK), the Drosophila Bicoid gene, the human thioredoxin gene, heat stable antigen and soybean 10A5 gene.

Iron responsive elements and iron regulatory protein binding sites may also advantageously be incorporated into the instant vectors to modulate RNA stability and particularly, translational efficiency. Histone RNAs, particularly their 3'UTRs, are especially useful for modulating RNA stability in a cell-cycle dependent fashion.

Also contemplated are modifications to or permutations of the elements listed above. The term "tandem copies" allows for both duplication and/or non-duplication of one or more of the outer nucleotides. For example, tandem copies of the pentamer AUUUA, includes sequences such as AUUUAUUUAUUUA as well as AUUUAAUUUAAUUUA.

RNA destabilising elements may be identified and or modifications made thereto using a computational approach and database analysis (Dandekar T *et al*).

Accordingly, biologically active fragments as well as variants and derivatives of reference destabilising elements are encompassed by the present invention.

Eukaryotic expression vectors are contemplated.

In a related embodiment the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable

polynucleotide; wherein said RNA element is a stabilising element which increases the stability of said transcript.

In another related embodiment the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a stabilising element which increases the stability of said transcript, wherein said stabilising element is, or is derived from, a gene selected from *alpha2 globin, alpha1 globin, beta globin, or growth hormone*, which are examples of long-lived mRNAs. As used herein, underscoring or italicising the name of a gene shall indicate the gene, in contrast to its protein product, which is indicated by the name of the gene in the absence of any underscoring or italicising. For example, "*alpha2 globin*" shall mean the *alpha2 globin* gene, whereas "alpha2 globin" shall indicate the protein product of the "*alpha2 globin*" gene.

The ability to destabilise a transcript and reduce the amount of protein produced by a cell will clearly be useful for a wide range of applications.

Another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript.

In another aspect, the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript, wherein the sequence of nucleotides encoding said destabilising element is, or is derived from, a gene selected from *c-fos, c-jun, c-myc, GM-CSF, IL-3, TNF-alpha, IL-2, IL-6, IL-8, IL-10, Urokinase, bcl-2, SGLT1 (Na(+)-coupled glucose transporter),*

Cox-2 (cyclooxygenase 2), IL-8, PAI-2 (plasminogen activator inhibitor type 2), beta1-adrenergic receptor or GAP43.

In one particular embodiment the polynucleotide sequences encoding the RNA destabilising elements are linked to sequences encoding a protein of interest, which in turn is linked to a promoter of interest that is preferably modulatable (i.e., inducible or repressible) such that expression is turned on and then off modulation . In this application, the RNA destabilising elements typically serve to shorten the period of expression of a functional mRNA or protein. This may be applied *in vitro* or *in vivo*. For example, a cell cycle-specific promoter could be combined with the RNA instability elements to express a protein of interest, exclusively in certain stages of the cell cycle. The protein of interest may be a functional protein or a reporter protein. In the latter example, reporter levels can be used as an indicator of cell-cycle stage or cell proliferation.

Yet another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript, wherein the sequence of nucleotides encoding said destabilising element is selected from any one of SEQ ID NOS 1 to 23, or biologically active fragments thereof, or variants or derivatives of these.

Even another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript, wherein the sequence of nucleotides encoding said destabilising element is set forth in SEQ ID NO: 1, 2 or 22 or biologically active fragments, variants or derivatives thereof.

Another aspect of the present invention contemplates an expression vector comprising a

transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element wherein said RNA element is a destabilising element which reduces the stability of said transcript and wherein said transcribable polynucleotide comprises a polynucleotide of interest and encodes a polypeptide.

One particular application is in the area of determining gene expression. Specifically, by reducing the amount of transcript produced in a cell it is possible to more accurately determine promoter or enhancer activity. In this application a reporter gene is used to determine promoter activity either directly, or indirectly as a fusion protein with another polypeptide whose expression has been modulated by regulatory elements within the vector.

In one embodiment the RNA destabilising sequences are incorporated into the region encoding the 3'-UTR of the reporter mRNA. Alternatively or in addition, destabilising elements are incorporated into the 5'-UTR and/or protein coding region, which is preferably not essential to, or does not interfere with, the selected activity of the encoded protein.

In a related embodiment the RNA destabilising sequences are used to destabilise a gene of interest when for example there is a need to accurately monitor or reduce its expression. Typically for this application, RNA destabilising elements are used in conjunction with reporter protein destabilising elements.

The subject expression vectors have applications in a variety of gene expression systems where it is preferable to have a brief period of mRNA or protein expression or where it is preferable to minimise the time lag between changes in promoter activity and the resultant changes in mRNA/protein levels.

Accordingly, yet another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element wherein said RNA element is a destabilising element which reduces the stability of said transcript and wherein said transcribable polynucleotide comprises a polynucleotide of interest and encodes a reporter polypeptide.

Even yet another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element wherein said RNA element is a destabilising element which reduces the stability of said transcript and wherein said transcribable polynucleotide comprises a sequence of nucleotides encoding a reporter polypeptide.

The expression vectors are designed for use in eukaryotic cell systems. It should be noted however that the RNA destabilising elements may be used in a wide range of eukaryotic and/or plants systems including cells, tissues or whole organisms defined as yeast, insect, nematode, fish, bird or mammal. For use in plants, different promoters and possibly different reporters and RNA destabilising elements (e.g., DST sequences) may be used.

It is contemplated that the expression vectors of the present invention will incorporate standard protein reporter molecules or destabilised reporter protein molecules. Standard reporter molecules are well known in the art.

Another aspect of the present invention contemplates the combination of a protein destabilising element (e.g., a DNA/RNA sequence encoding an intracellular protein degradation signal or degron which may be selected from a destabilising amino acid at the amino-terminus of a polypeptide of interest, a PEST region or a ubiquitin) and an mRNA destabilising element (e.g., multiple copies of the nonamer UUAUUUAUU), such that both mRNA and protein are destabilised. For example, one such embodiment incorporates into an expression vector, a PEST sequence immediately upstream of the translation stop codon and 4 nonamers located downstream of the stop codon (preferably 20nt or more from stop codon).

In this way, reporter protein may be destabilised both at the protein level and the mRNA level.

The destabilised reporter protein may be any suitable protein. For example, destabilised GFP proteins are suitable, such as for example d1EGFP, d1EYFP and d1ECFP comprising the d1

mutant of MODC. The destabilised luciferase protein has been described by Leclerc G. *et al.* The MODC PEST sequence was used. The MODC from d1EGFP is also contemplated.

Any method of destabilising a polypeptide of interest is contemplated by the present invention. For example, a polypeptide of interest can be modified to include a destabilising amino acid at its amino-terminus so that the protein so modified is subject to the N-end rule pathway as disclosed, for example, by Bachmair *et al* in U.S. Patent Serial No. 5,093,242 and by Varshavsky *et al.* in U.S. Patent Serial No. 5,122,463. In a preferred embodiment of this type, the destabilising amino acid is selected from isoleucine and glutamic acid, more preferably from histidine tyrosine and glutamine, and even more preferably from aspartic acid, asparagine, phenylalanine, leucine, tryptophan and lysine. In an especially preferred embodiment, the destabilising amino acid is arginine. In some proteins, the amino-terminal end is obscured as a result of the protein's conformation (i.e., its tertiary or quaternary structure). In these cases, more extensive alteration of the amino-terminus may be necessary to make the protein subject to the N-end rule pathway. For example, where simple addition or replacement of the single amino-terminal residue is insufficient because of an inaccessible amino-terminus, several amino acids (including lysine, the site of ubiquitin joining to substrate proteins) may be added to the original amino-terminus to increase the accessibility and/or segmental mobility of the engineered amino terminus.

Modification or design of the amino-terminus of a protein can be accomplished at the genetic level. Conventional techniques of site-directed mutagenesis for addition or substitution of appropriate codons to the 5' end of an isolated or synthesised antigen-encoding polynucleotide can be employed to provide a desired amino-terminal structure for the encoded protein. For example, so that the protein expressed has the desired amino acid at its amino-terminus the appropriate codon for a destabilising amino acid can be inserted or built into the amino-terminus of the protein-encoding sequence. Where necessary, a nucleic acid sequence encoding the amino-terminal region of a protein can be modified to introduce a lysine residue in an appropriate context. This can be achieved most conveniently by employing DNA constructs encoding "universal destabilising segments". A universal destabilising segment comprises a nucleic acid construct which encodes a polypeptide structure, preferably segmentally mobile,

containing one or more lysine residues, the codons for lysine residues being positioned within the construct such that when the construct is inserted into the coding sequence of the antigen-encoding polynucleotide, the lysine residues are sufficiently spatially proximate to the amino-terminus of the encoded protein to serve as the second determinant of the complete amino-terminal degradation signal. The insertion of such constructs into the 5' portion of a antigen-encoding polynucleotide would provide the encoded protein with a lysine residue (or residues) in an appropriate context for destabilisation.

In another embodiment, the polypeptide of interest is modified to contain a PEST region, which is rich in an amino acid selected from proline, glutamic acid, serine and threonine, which region is optionally flanked by amino acids comprising electropositive side chains. In this regard, it is known that amino acid sequences of proteins with intracellular half-lives less than 2 hours contain one or more regions rich in proline (P), glutamic acid (E), serine (S), and threonine (T) as for example shown by Rogers *et al.* (1986, *Science* **234** (4774): 364-368). In yet another embodiment, the polypeptide of interest is conjugated to a ubiquitin or a biologically active fragment thereof, to produce a modified polypeptide whose rate of intracellular proteolytic degradation is increased, enhanced or otherwise elevated relative to the unmodified polypeptide.

Still another aspect of the present invention contemplates an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript, wherein said reporter protein is selected from Luciferase, Green Fluorescent Protein, Red Fluorescent Protein, SEAP, CAT, or biologically active fragments thereof, or variants or derivatives of these.

Such vectors can be used to screen for drugs or treatments that alter the activity of that promoter. Compared to existing reporter vectors, a near "real-time" measurement of drug action can be obtained.

Still another aspect of the present invention is directed to an expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript and wherein said vector comprises one or more members in any order selected from the group consisting of:

(i) a multiple cloning site for introducing a sequence of nucleotides, which site is preferably cleavable enzymatically or otherwise biochemically to provide a linearised vector into which PCR amplification products are clonable directly (e.g., an Ec1HK1 site);

(ii) a reporter gene;

(iii) a promoter and/or enhancer for regulating expression of said transcribable polynucleotide;

(iv) a polyadenylation sequence;

(v) a selectable marker gene; and

(vi) an origin of replication.

Another aspect of the present invention contemplates vectors or sets of vectors, particularly but not exclusively, plasmids, with applications in the study or measurement or monitoring of gene expression (e.g., promoter activity). Many other vectors could also be used such as for example viruses, artificial chromosomes and other non-plasmid vectors.

One embodiment involves pairs or sets of plasmids, each containing one or more of the mRNA destabilising sequences described above incorporated into a construct encoding a destabilised reporter protein such as, for example, d1EGFP, d1EYFP, d1ECFP or d1DsRed. One plasmid

(the control) from each pair or set contains a promoter 5' of the reporter encoding region. The promoter is comprised of elements which is modulatable (i.e., inducible or repressible) by exogenous treatments (e.g., the TRE combined with a minimal promoter such as mCMV; see Fig. 2c). Alternatively, a constitutively active promoter such as TS, SV40, CMV, TK or RSV is used (see Fig. 2b). In plant systems the Top-ten promoter could replace TRE, and the 35S promoter of cauliflower mosaic virus can replace SV40 etc. Agrobacterium tumefaciens can be used in plants to facilitate gene transfer. The other plasmid(s) in the pair or set are identical to the control plasmid, except that a cloning site (MCS) replaces the promoter, and the reporter encoding region encodes a reporter similar to but distinguishable from the control reporter (see Fig. 2a). In a preferred embodiment, the control plasmid encodes a destabilised variant of EGFP (e.g., d1EGFP, d1EYFP or d1ECFP) and the other vectors (test vectors) each encode a different colour variant from the same list (same protein half-life).

In other embodiments, a control and one of the test reporters are incorporated into a single vector, such as for example a bi-directional plasmid (see Fig. 3).

In the above embodiments, both control and test plasmids encode a destabilised mRNA, which in turn encodes a destabilised protein. Thus the time lag between decreased promoter activity and decreased reporter protein levels, is significantly reduced compared to the time lag with existing constructs. Similarly, increased promoter activity is more readily and quickly detectable due to the reduced levels of pre-existing mRNA and protein. Other differences between the control and test constructs, which can lead to errors, are minimised by using fluorescent proteins that differ from each other by only a few small mutations. Compared to luciferase or other enzyme based assays, the fluorescent reporters described here, offer several other advantages including:

- Several different reporters can be measured in the same cells/samples.

- Live cells can be measured, allowing multiple time points of the same samples or further manipulation post-measurement e.g., measurement of the same cells before and after treatment with a drug.

- Successfully transfected cells can be visualised by fluorescent microscopy. Therefore poor transfections can be identified simply by looking at the cells under a microscope, without further investment of resources.

- No substrates are required, therefore the method is less technically demanding, faster, less expensive and more accurate.

- Both control and test reporter expression can be measured simultaneously by flow cytometry (see advantages of flow cytometry below).

- Embodiments utilising TREs as the control promoter can only be used in Tet-On or Tet-Off cell lines, but as compared to other control promoters, exhibit less interference from or to the test promoter and are less affected by various stimuli used to examine inducibility of the test promoter. Thus, they provide a more accurate measurement of transfection efficiency and relative test promoter activity. Control reporter expression can be switched on or off as required and used to confirm the lack of promoter cross-talk or compensate for it if present.

- In another embodiment, the control and one of the test reporters described above are both incorporated into a single vector, preferably a bi-directional plasmid. Interference between the two promoters, which is a major drawback of previous dual promoter vectors, is minimised by using TREs in the control promoter. Such a single vector system prevents the inaccuracies of co-transfection studies.

The invention also provides vectors in which informative promoters or promoter fragments, are placed upstream of the reporter-encoding region. The present invention provides a simpler, quicker and more cost-effective reporter system for such assays when using utilising EGFP variants as opposed to luciferase or other enzymes as the reporter. Furthermore, the inclusion of mRNA instability elements allows a near real-time analysis.

Informative promoters include, but are not restricted to, cell cycle-dependent promoters (e.g., cyclin A, B, or D1, histone or topoisomerase I promoters), promoters activated by apoptotic (cell death) pathways and promoters/fragments linked to mitogenic signals (Table 1). Examples of informative enhancers that can be used include any of those used in Clontech's Mercury Pathway Profiling Systems. Clontech's Mercury In Vivo Kinase Assay Kits represent another example of how the present invention can be used. In this example the promoter element is a TRE that is combined in cells with a chimeric TetR-transactivator protein that permits transcription from the TRE only when a specific kinase is active and can phosphorylate the transactivator domain of the fusion protein. Thus, the present invention can be used to provide a more real-time measurement of specific kinase activity.

Even still another aspect of the present invention contemplates a cell transfected or transduced with a vector according to the present invention comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide.

In some applications the expression vectors or cells expressing the reporter constructs are inserted into an organism to allow measurement of reporter activity *in vivo*. In some of these applications, destabilised luciferase rather than destabilised EGFP variants may be the preferred reporter. For example, transgenic mice expressing destabilised luciferase under the control of an informative promoter, can be used to measure the activity of that promoter in the tissues of a live mouse, using a photon camera (photon camera analysis is described by Contag, *et al*, 1997). The mRNA destabilising sequences serve to improve the temporal correlation between promoter activity and reporter levels, thus providing a significant improvement to applications such as drug screening, which benefit from a near real-time measurement of promoter activity.

In some applications it is desirable to express, either *in vitro* in cell-based systems or *in vivo* in mammalian systems, both a reporter molecule and a functional gene product. This may involve two separate mRNAs, each containing an mRNA destabilising element. Alternatively, an mRNA destabilising elements may be incorporated into a single destabilised transcript that gives rise to two separate proteins (e.g., using an internal ribosome entry site; IRES) or a fusion

protein comprised of the reporter and the functional gene product.

The invention also provides cell lines stably expressing these vectors (with or without a control). Such cells have applications in areas such as drug screening. For example, cells containing a MAPK-dependent reporter vector provide a rapid and inexpensive method for testing the efficacy of drugs designed to inhibit MAPK or any pathway upstream of MAPK-dependent transcription in those cells. In SKBR3 human breast cancer cells, for example, MAPK activity is dependent on signalling from the overexpressed ErbB2 protein. Therefore, drugs that inhibit ErbB2, would cause a decrease in the fluorescence of SKBR3cells containing such a construct but not in cells lacking ErbB2. Alternatively, cells could be tested ± drug and ± a specific ligand or treatment that leads to MAPK activation via a different pathway, in order to monitor inhibition of that pathway. Cell lines (or organisms) stably expressing a vector linked to a cell-cycle-regulated promoter can be used as very fast, simple and inexpensive means for measuring cell-cycle progression or cell proliferation. Such cell lines have obvious utility in drug screening and are contemplated in the present invention. Examples of cell-cycle regulated promoters are readily available, for example, (Lee, H *et al.* 1995), (Stein, J *et al.* 1996) and (Huet, X *et al.* 1996).

Another embodiment of the present invention includes vectors, for the study of post-transcriptional regulation, particularly mRNA stability. The reporter is, for example, a destabilised variant of EGFP (e.g., d1EGFP, d1EYFP, d1ECFP), with a different color variant in each separate vector. The TRE (linked to a minimal promoter such as mCMV) is 5' of the reporter encoding region and drives transcription in a tetracycline (or doxycycline) dependent fashion. Other inducible promoter systems can also be used.

In one embodiment, the mRNA instability elements described above are not included and in their place, MCSs are located, primarily in the 3'-UTR (see Fig. 4a) but also in the 5'-UTR and/or coding region in some specific embodiments. Sequences thought to affect mRNA stability can be tested by cloning them into the appropriate cloning site of a vector containing one color variant and measuring the rate of decrease in reporter levels after blocking transcription with tetracycline or doxycycline (see Fig. 7). If desired, the rate of decay can be

compared between the "test vector" and the "control vector," (which encodes a different colour EGFP variant and does not contain the sequence being tested) in the same cells.

The MCS may usefully comprise or work in conjunction with restriction endonuclease sites which allow direct cloning of PCR products having overhangs (see below).

In another related embodiment of the invention, one or more mRNA instability element(s) are included to assist scientists specifically searching for mRNA stabilising elements. Similarly, other embodiments include mRNA stabilising element(s) to assist scientists specifically searching for mRNA destabilising elements.

In other embodiments, the control and one of the test reporters are both incorporated into a single vector, preferably a bi-directional plasmid (see Fig. 4b).

Stabilising elements are useful for increasing levels of expressed protein for example during protein purification where high levels or protein are required or when a promoter is weak.

Interference between the two promoters and moreover, transcription effects of the element or various stimuli tested, is circumvented by using a TRE or similar element to drive both reporters and by measuring reporter levels after addition of doxycycline (or tetracycline), which shuts off transcription from the vector.

Still another aspect of the present invention contemplates a cell transfected or transduced with a vector according to the present comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript.

A related aspect of the instant invention considers a genetically modified non-human organism comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said

transcribable polynucleotide; wherein said RNA element is a destabilising element which reduces the stability of said transcript.

Yet a further embodiment of the present invention contemplates a method for determining expression of a polynucleotide of interest, said method comprising expressing said polynucleotide of interest from a reporter expression vector for a time and under conditions sufficient for RNA and protein synthesis to occur, said vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding a transcribed element and said polynucleotide of interest wherein said transcribed element modulates the stability of a transcript corresponding to said transcribable polynucleotide; and wherein said expression vector comprises one or more members in any order selected from the group consisting of:

(i) a multiple cloning site for introducing said polynucleotide of interest;

(ii) a reporter gene;

(iii) a promoter for regulating expression of said polynucleotide of interest and/or a reporter gene, which promoter is preferably modulatable (e.g., using a tetracycline responsive element (TRE));

(iv) a polyadenylation sequence;

(v) a selectable marker gene; and

(vi) an origin of replication;

and measuring the level or activity of the reporter over time compared to a control wherein said destabilising element enhances the temporal correlation between promoter or enhancer activity and reporter level or activity.

A combination of different RNA destabilising elements acting in combination is contemplated herein.

The present invention is further described by the further non-limiting Examples.

EXAMPLE 1
Cloning DNA Elements into Vectors

Cloning is carried out according to existing methods, using restriction enzyme sites in the MCS or direct ligation of PCR products in the case of vectors with a "T overhang" in the MCS. With respect to post-transcriptional reporter vectors, however, the inclusion of a MCS in the 3'-UTR or other regions is a significant improvement over current vectors, which were designed for transcriptional or other studies and do not contain convenient cloning sites in these locations.

EXAMPLE 2
Transfection

Co-transfection of control and test vectors is performed as per existing methods (e.g., Fugene [Boehringer Mannheim, Mannheim, Germany] or electroporation), except in the case of the single (e.g., bi-directional) vector systems described above, which require only one vector and thus eliminate inaccuracies associated with co-transfection.

EXAMPLE 3
Measurement of Reporter Expression

An immediate advantage of the vGFP system is that reporter expression can be visualized directly in living cells, simply by viewing the tissue culture plate or flask under a fluorescent microscope. Therefore, poor transfections can be identified and discarded before any additional time is wasted. Quantitative measurement can be performed using a fluorometer (e.g., 96 well

plate format) and since live cells can be measured, the same samples can be measured repeatedly e.g., in a time course.

A further advantage compared to luciferase and other enzyme based assays is that flow cytometry can also be used to measure reporter levels.

EXAMPLE 4

Advantages of Using Flow Cytometry to Measure Reporter Levels

i. Two or more reporters (control and test) as well as additional parameters, can be measured individually in every cell at a rate of >2,000 cells per second. Therefore, in this application, the method yields thousands to hundreds of thousands of data points per sample versus one datum point for existing luciferase assays.

ii. Accurate measurement of transfection efficiency: This is useful for optimising transfection protocols. In addition to allowing comparison of different methods, it is also possible to measure both expression per cell and the proportion of cells expressing. This helps the investigator to determine the cause of any problems.

iii. Identification of co-transfection errors: Co-transfection studies are based on the premise that cells will take up and express an amount of control reporters, which is proportional to the amount of test plasmid taken up by the same cells. This is not always the case. By using the flow cytometry method described here, it is possible to correlate test versus control expression levels in different cells of the same sample. Invalid samples can be identified by the lack of a good linear relationship between test and control reporter levels. Such errors go unnoticed in current methods.

iv. Simultaneous measurement of additional parameters: Fluorescent labelled antibodies can be used to quantify specific proteins on a cell by cell basis and this can be correlated with reporter levels to determine whether that protein affects gene expression via the element cloned into the reporter construct. Alternatively, the protein of interest can be expressed as a vGFP-fusion protein (the protein of interest fused to a GFP

variant) via transfection of an appropriate expression vector (inducible or non-inducible). Levels of the specific protein can then be correlated with the expression of a different GFP variant linked to a regulatory element of interest (co-transfected or transfected at a different time). In a third application, the vGFP reporter is linked to regulatory elements (e.g., promoters) thought to be cell cycle specific. Transfected cells are stained with a fluorescent DNA dye such as propidium iodide to measure DNA content, which is then correlated with reporter expression. In principle, several of the DNA constructs described herein, each containing a different vGFP, could be co-expressed and independently measured. Furthermore, other fluorescent markers could be used in conjunction with these vectors (singly or in multiples).

v. <u>Cell Sorting:</u> Using a cell sorter, it is possible to isolate viable vGFP expressing cells from the non-expressors. This technique can be used to select stably expressing cells or to remove non-expressors prior to assay initiation. Similarly, it is possible to remove cells expressing very low and/or very high levels of vGFP. This can be used to generate a more homogeneous population and/or to remove cells expressing levels so high that they may not be physiological relevant or may perturb normal cellular function and/or may otherwise adversely affect the data obtained from the DNA vectors described herein.

It is important to note that transient and stable transfections of expression vectors result in a cell population with very heterogeneous levels of expression. In general a thousand fold difference between the highest and lowest expressor is not unusual. The present invention not only offers a method for selecting homogeneous populations when required (see v above), but can also utilise heterogeneity to the benefit of the scientist. For example, identifying co-transfection errors. Another example of this relates to (iv) above. To determine whether protein X affects transcription from promoter Y, then cells are transfected with a reporter construct expressing d1EGFP under the control of promoter Y. If required, cell sorting can be used to isolate cells transiently or stably expressing appropriate levels of d1EGFP. These cells are in turn transiently transfected with a vector expressing a protein X-EYFP fusion protein. During flow cytometry, EGFP is plotted on one axis and EYFP on the other. A positive correlation

would indicate that protein X increases transcription from promoter Y and a negative correlation would indicate that protein X inhibits transcription from promoter Y.

Currently, scientists attempting to establish such a correlation would select several different clones of high versus low expressors of protein X. Each clone would then be separately transfected with a promoter Y-luciferase construct and the luciferase activities measured. The use of cell clones requires months of preparation and introduces many variables including pre-existing heterogeneity amongst the host cells and variable sites of vector integration (vector DNA may interfere with a specific gene at the integration site and this site is different for every clone). Furthermore, such a method yields very few data points, with each datum point obtained from a different transfection of a different clone. Thus, the new system is not only more versatile but is quicker and more accurate than existing methods.

EXAMPLE 5

Laser scanning cytometry (LSC)

Unlike flow cytometry, LSC measures multi-colour fluorescence and light scatter of cells on slides, and records the position and time of measurement for each cell analysed. This technique provides data equivalent to flow cytometry but has the advantage of being microscope slide based (Darzynkiewicz *et al.*, 1999; Kamentsky *et al.*, 1997). Owing to the fluorescence of GFP and its variants, the techniques described for flow cytometry are also applicable to LSC.

EXAMPLE 6

Specific Methods for Post-Transcriptional Assays

These are best summarised by using the example of a study aimed at determining whether a specific 3'-UTR fragment affects mRNA stability. Although this example is one of transient expression, stable transfection could also be used

(i). The 3'-UTR fragment is ligated into the 3'-UTR cloning site of the test vector and co-transfected with the control vector into a Tet-Off cell line. In the case of the bi-directional vector, no control vector is required. Indeed, the typical application does not

require a control reporter or vector since rate of decay can be measured in samples from within the same transfection. 5'-UTR fragments can be tested by inserting them into vectors with a 5'-UTR cloning site.

(ii). The cells are grown in the absence of doxycycline (or tetracycline) for 6-48 h to allow expression of both vectors. Alternatively, cells are grown with low doses of doxycycline (or tetracycline), for 6-48 h to block transcription and then switched to medium without doxycycline (or tetracycline) for 2-12 h to provide a brief burst of transcription.

(iii). High doses of doxycycline (or tetracycline) are then applied to shut off transcription from both vectors.

(iv). The fluorescence of both reporters is measured (by flow cytometry, fluorometry or LSC) in a time course following addition of doxycycline (or tetracycline).

If the cloned element confers mRNA instability, a more rapid decrease in "test" fluorescence will be seen compared to "control" fluorescence of the same cells or sample. Similar studies can be used to test an mRNA element's response to certain stimuli or its effect in different cells or cells expressing different amounts of a specific protein, such as an RNA-binding protein. Applying the stimulus after doxycycline will determine whether pre-existing transcripts are affected by the stimulus. Inserting the element in different locations (e.g., 5'-UTR, 3'-UTR) will determine whether its function is dependent on position. Inserting a protein/polypeptide coding sequence (in frame) within the reporter-coding region of the vector, can be used to determine the effect of that sequence on mRNA and protein stability.

RNA can be extracted from transfected cells and used to measure reporter mRNA directly.

EXAMPLE 7

Transcription Reporter Vectors

The vectors are plasmids suitable for expansion in *E. coli* and expression of a fluorescent reporter in eukaryotic cells. The plasmids may be used in sets. Each set is comprised of one or more "control" vectors and one or more "test" vectors. Every vector within a set expresses a similarly destabilised mRNA and a similarly destabilised fluorescent reporter protein. In addition to the standard features of such plasmids (ampicillin resistance, origin of replication etc.), each plasmid contains the following construct (see also Figs 2 and 3):

5'---- MCS/promoter----transcription start site---5'UTR---ATG--vEGFP encoding region --stop codon—3'UTR with mRNA destabilising element---polyadenylation signal

Where:

MCS/promoter denotes either a multiple cloning site (test vectors; see Fig. 2a) or a constitutively active promoter such as SV40 (control vectors; see Fig. 2b) or an inducible promoter such as TRE-mCMV (control vector; see Fig2c).

ATG denotes a translation start codon.

Stop codon denotes a translation stop codon.

5'UTR denotes a 5' untranslated region.

3'UTR with mRNA destabilising element denotes a 3' untranslated region containing one or more of the mRNA destabilising elements outlined.

vEGFP denotes a destabilised variant of EGFP. One set of plasmids is provided for each type of destabilising modification (e.g., 1 hr half-life, 2 hr half-life). Within each set of plasmids,

one vector is provided for each different colour variant. For example, one set contains vectors expressing d1EGFP, d1EYFP, d1ECFP whereas another set expresses the d2 variants.

In other examples, the control and one of the test reporters described above are both incorporated into a single vector, preferably a bi-directional plasmid (see Fig. 3).

EXAMPLE 8

Post-transcription Reporter Vectors

Similar to the transcription reporter "control" vectors that contain a TRE-mCMV promoter , except that the mRNA destabilising element in the 3'-UTR is replaced with a MCS (see Fig. 4a). In some embodiments, MCS are also located in the 5' UTR and/or coding region.

Such a construct can be used as a "test" or a "control" vector for the post-transcriptional assays outlined herein.

In other examples, the control and one of the test reporters described above are both incorporated into a single vector, preferably a bi-directional plasmid (see Fig. 4b).

EXAMPLE 9

Reporter Vectors for Assaying Specific Pathways

Vectors similar to those described herein, into which a regulatory element has been inserted into the MCS for the purpose of studying or measuring the function of said regulatory element. For example, plasmids similar to the transcription reporter plasmids outlined herein, except that they contain within the MCS, a promoter or promoter element(s) or enhancer(s) that are responsive to pathways such as those referred to in Table 1 and/or contain any of the following cis-acting enhancer elements as described in Clontech's Mercury Pathway Profiling Systems: AP1, CRE, E2F, GRE, HSE, ISRE, Myc, NFAT, NFκB, p53, Rb, SRE. The reporter is preferably a destabilised version of GFP, luciferase or SEAP.

Cell lines and Mice for Assaying Specific Pathways

Cell lines or genetically modified mice stably expressing one or more of the vectors described herein.

EXAMPLE 10
Method of Use

The vectors described in this invention are used for experimentation in essentially the same manner as the existing vectors that they replace, with the exception of the new methods described herein.

Method of Construction

The vectors and DNA constructs outlined here are assembled using standard cloning techniques. The SV40 and TRE-mCMV promoters described here as well as the more standard components of plasmid vectors (e.g., origin of replication, antibiotic resistance or another selection gene) are readily available in a variety of common vectors. DNA sequences encoding the destabilised variants of EGFP (e.g., d1EGFP, d1EYFP, d1ECFP and d2EGFP, d2EYFP, d2ECFP) are available from Clontech (Clontech Laboratories Inc., Palo Alto, CA, USA). DNA sequences encoding destabilised DsRed variants are constructed by fusing to the 3' end of the DsRed encoding region, sequences encoding the degradation domains (or mutants thereof) from short-lived proteins. For example, amino acids 422-461 from mouse ornithine decarboxylase, which contains a PEST sequence. Such sequences could potentially be derived from existing dEGFP variants.

EXAMPLE 11

Summary

In summary the present vectors and methods are now available:

- Expression vectors or parts thereof that incorporate one or more mRNA instability elements in order to provide a relatively short-lived mRNA. Compared to existing expression vectors, the vectors claimed here provide kinetics of protein expression that correlate more closely with promoter activity. For example, the time lag between decreased promoter activity and decreased mRNA and protein levels is substantially reduced.

- Expression vectors or parts thereof encoding a destabilised mRNA that in turn, encodes a destabilised protein. Compared to existing vectors, the vectors claimed here provide kinetics of protein expression that correlate more closely with promoter activity.

- Expression vectors or parts thereof in which the mRNA destabilising elements are comprised of sequences cloned from short-lived mRNAs such as *c-fos,* examples of short-lived mRNAs include; c-fos, c-myc, GM-CSF, IL-3, TNF-alpha, IL-2, IL-6, IL-8, Urokinase, bcl-2, SGLT1 (Na(+)-coupled glucose transporter), Cox-2 (cyclooxygenase 2), IL8, PAI-2 (plasminogen activator inhibitor type 2), beta1-adrenergic receptor, GAP43 (5'UTR and 3'UTR) AU-rich elements (AREs) and/or U-rich elements, including but not limited to single, tandem or multiple or overlapping copies of the nonamer UUAUUUA(U/A)(U/A) (where U/A is either an A or a U) (Lagnado *et al* 1994) and/or the pentamer AUUUA (Xu *et al* 997) and/or the tetramer AUUU (Zubiaga *et al.* 1995). Also included are minor modifications to or permutations of the elements listed above. The term "tandem copies," allows for both duplication and/or non-duplication of one or more of the outer nucleotides. For example, tandem copies of the pentamer AUUUA, includes sequences such as AUUUAUUUAUUUA as well as AUUUAAUUUAAUUUA. The 3' UTR or 5' UTR regions of short-lived mRNAs often contain destabilising sequences.

- Expression vectors or parts thereof in which the mRNA destabilising elements were identified or validated using the vectors described herein, which provide substantially improved methods for identifying such elements.

- Expression vectors or parts thereof, in which the destabilised mRNA encodes a short-lived reporter protein such as a destabilised variant of EGFP or luciferase. Compared to existing reporter vectors, the vectors claimed here provide kinetics of reporter expression that correlate more closely with promoter activity. For example, the time lag between decreased promoter activity and decreased mRNA and protein levels is substantially reduced.

- Sets of reporter vectors or parts thereof that encode similarly destabilised mRNAs (similar to other vectors in the same set), which in turn, encode similarly (similar to other vectors in the same set) destabilised variants of EGFP or DsRed or other fluorescent markers. One or more vectors (control vectors) within each set contain a constitutive promoter (e.g., SV40, CMV, RSV, TK, TS; see Fig. 2b) or an inducible promoter (e.g., TRE-mCMV; see Fig. 2c), whereas the other vectors (test vectors) within each set contain a cloning site (e.g., MCS) in place of the promoter (e.g., see Fig. 2a). Applications of these vectors include but are not limited to the study or measurement of promoter activity. For example, a promoter element of interest can be cloned into the MCS of a test vector encoding d1EGFP and reporter expression measured relative to that of a control vector expressing d1EYFP. Also claimed is each individual vector described well as bi-directional vectors or other single vector systems that incorporate one test and one control reporter construct within the same vector (e.g., Fig. 3a and Fig. 3b). Compared to existing sets of reporter vectors, the vector sets claimed here offer the following advantages:

  a). A measurement of promoter activity that is closer to real-time.

b). Decreased errors due to the closer similarity between control and test constructs.

c). Decreased errors resulting from cross talk between test promoters and the control promoters. By utilising inducible promoters in the control vectors, such cross talk is minimised and/or identified and corrected for via measurement with and without induction.

d). Can be used in conjunction with the flow cytometry/LSC methods described.

- Reporter vectors or sets of reporter vectors or parts thereof that utilise an inducible promoter, preferably but not exclusively the tetracycline responsive element (TRE), to drive expression of a destabilised fluorescent reporter protein (preferably but not exclusively destabilised EGFP variants). Such vectors contain cloning sites in the 3'-UTR (e.g., Fig. 4a) and/or 5'-UTR and/or reporter coding region, such that regulatory elements or putative regulatory elements can be cloned into a vector expressing one color fluorescent reporter and, if required, compared to a control vector which expresses a different color reporter and does not contain the element of interest. Such vectors have applications in the study or measurement of post-transcriptional regulation, since transcription can be shut off as desired via the inducible promoter. The advantages offered by these vectors include those listed in b-d, the ability to separate post-transcriptional effects from transcriptional effects and also:

  a). incorporation of convenient cloning sites, not present in other vectors; and

  b). the technique is more rapid than any existing method.

- Single vector systems that essentially link one test and one control construct and described (e.g., Fig. 4b). Both test and control reporters are driven by an inducible promoter and the cloning sites allow ligation of regulatory elements into the test construct only. In addition to the advantages of vectors outlined, the single vector

systems eliminate problems and inaccuracies associated with co-transfection of separate test and control vectors.

- The use of flow cytometry or LSC to measure the levels of 2 or more fluorescent reporters expressed via the vectors outlined. In this application, the method yields thousands to hundreds of thousands of data points per sample versus one datum point for existing enzyme-based assays. Two or more reporters (control and test) as well as additional parameters (e.g., DNA content, levels of other proteins) can be measured individually in every cell. Also encompassed is the use of flow cytometry to correlate the levels of 2 or more reporters in multiple cells within the same sample and the utilisation of such data to optimise transfection protocols and/or identify problems associated with co-transfection. For example, invalid samples can be identified by the lack of a good linear relationship between test and control reporter levels. Such errors go unnoticed in current methods.

- Methods for utilising the post-transcriptional reporter vectors claimed. These methods are best summarised by using the example of a study aimed at determining whether a specific 3'-UTR fragment affects mRNA stability. Although this example is one of transient expression, stable transfection could also be used.

(i). The 3'-UTR fragment is ligated into the 3'-UTR cloning site of the test vector and co-transfected with the control vector into a Tet-Off cell line. In the case of the single vector system, no control vector is required. 5'-UTR fragments can be tested by inserting them into vectors with a 5'-UTR cloning site.

(ii). The cells are grown in the absence of doxycycline (or tetracycline) for 6-48 h to allow expression of both vectors. Alternatively, cells are grown with low doses of doxycycline (or tetracycline), for 6-48 h to block transcription and then switched to medium without doxycycline (or tetracycline) for 2-12 h to provide a brief burst of transcription.

(iii). High doses of doxycycline (or tetracycline) are then applied to shut off transcription from both vectors.

(iv). The fluorescence of both reporters is measured (by flow cytometry, fluorometry or LSC) in a time course following addition of doxycycline (or tetracycline).

If the cloned element confers mRNA instability, a more rapid decrease in "test" fluorescence will be seen compared to "control" fluorescence of the same cells or sample. Similar studies can be used to test an mRNA element's response to certain stimuli or its effect in different cells or cells expressing different amounts of a specific protein, such as an RNA-binding protein. Applying the stimulus after doxycycline will determine whether pre-existing transcripts are affected by the stimulus. Inserting the element in different locations (e.g., 5'-UTR, 3'-UTR) will determine whether its function is dependent on position. Inserting a protein/polypeptide coding sequence (in frame) within the reporter protein-coding region of the vector can be used to determine the effect of that sequence on mRNA and protein stability.

RNA can be extracted from transfected cells and used to measure reporter mRNA directly.

- Cell lines transiently or stably expressing one or more of the expression constructs or parts thereof claimed.

- Cell lines transiently or stably expressing one or more of the expression constructs or parts thereof claimed, wherein the expression construct contains a regulatory element that serves as a marker for the activation of signal transduction pathways associated with human disease and/or response to drug treatment. Such pathways include, but are not restricted to the list in Table 1 and those indicated elsewhere in this document (e.g., CRE, SRE, AP1, cyclin A, B and D1 promoters).

- Transgenic mice, knock-in mice or other genetically modified mice expressing one or more of the expression constructs or parts thereof claimed.

- Transgenic mice, knock-in mice or other genetically modified mice expressing one or more of the expression constructs or parts thereof claimed, wherein the expression construct contains a regulatory element that serves as a marker for the activation of signal transduction pathways associated with human disease and/or response to drug treatment. Such pathways include, but are not restricted to the list in Table 1.

- Destabilised variants of DsRed or the mutant DsRed1-E5. These can be constructed by fusing to the C-terminus of DsRed, degradation domains (or mutants thereof) from various unstable proteins. For example, amino acids 422-461 of mouse ornithine decarboxylase, which contains a PEST sequence (Li *et al.* 1998). Additional destabilising elements can also be added. Also contemplated are DNA constructs encoding destabilised variants of DsRed.

- Vectors encoding destabilised variants of DsRed outlined, including such vectors also containing the mRNA instability elements outlined.

- The following method for creating Tet-Off or Tet-On cell lines:
  The tTA or rtTA expression vector, preferably a retrovirus, adenovirus or plasmid, is stably expressed in the cell line of interest using standard techniques and expressing cells are isolated via a drug resistance marker. These cells are then transiently transfected with a TRE-vGFP construct and subjected to several rounds of cell sorting by flow cytometry. For example, good Tet-Off cells would show no fluorescence in the presence of doxycycline and are sorted as such. After a further 5-48 hr without doxycycline, green cells are sorted. Finally, the cells are grown for a week or more without doxycycline and sorted a final time to eliminate stably transfected (green) cells.

EXAMPLE 12

Vectors Incorporating mRNA and Protein Destabilising Elements

The coding region of interest (e.g., a reporter such as EGFP or luciferase) could include combined sequence of a protein destabilising element (e.g., d1 mutant of MODC; Clontech, but also including other PEST sequences or other protein destabilising elements such as ubiquitination sites) and an mRNA destabilising element (e.g., AU-rich element).

For example, the stop codon of luciferase and DsRed is replaced with a Hind3 site (AAGCTT) to allow the addition of the sequence: AAGCTTAGCCATGGCTTCCCGCCGGCGGTGGCGGCGCAGGATGATGGCACGCTGC CCATGTCTTGTGCCCAGGAGAGCGGGATGGACCGTCACCCTGCAGCCTGTGCTTCT GCTAGGATCAATGTGTAG which is Clontech's d1 mutant of MODC that confers a 1 hr half life to EGFP. This is followed by a linker (which becomes part of the 3'UTR and then: UUAUUUAUU GGCGG UUAUUUAUU CGGCG UUAUUUAUU GCGCG UUAUUUAUU ACTAG which contains 4 nonamers and connects to the Xba1 site of the parent vector (pGL3; Promega) also in the 3'UTR but further downstream.

EXAMPLE 13

Direct Ligation of PCR Products

Inclusion into the MCS of a vector of two separate but nearby RE recognition sites, which, when cut with that/those RE(s), leave a 3' overhang of a single T nucleotide at both ends of the remaining vector. For example, the recognition sequence for EclHK1 is GACNNN, NNGTC (cuts between 3rd and 4th N from 5' leaving a 3' overhang of a single N at each end). Two of these sites are incorporating into the MCS, such that the short region between them is released by digestion with EclHK1, leaving a linearised vector with a 3' overhang of a single N at each end. In this example, the upstream recognition sequence should be 5'GACNNTNNGTC3' and the downstream sequence 5'GACNNANNGTC3'. After cutting with EclHK1, the large vector fragment will contain a single 3' T overhang at both ends (similar to Promega's pGEM-T Easy vector). This facilitates the direct ligation of PCR

products that are produced with a polymerase such as Taq, that yields a 5' A overhang. This constitutes a significant improvement over standard MCSs, which do not support direct ligation of PCR products without inclusion of RE sites into PCR primers and subsequent digestion of PCR product. This is also a significant improvement over the pGEM-T Easy vector, which cannot be amplified (supplied as linear) and is useful only for subcloning (i.e., PCR products are typically ligated into pGEM-T Easy, amplified and then removed by RE digestion and subsequently cloned into the expression vector of interest). Thus, the present MCS permits direct ligation of PCR products without the need for digesting them with a RE (which is often problematic) or subcloning them into an intermediate vector.

EXAMPLE 14

Destabilised Reporter Model Shows Improved Real-Time Analysis

Plasmid reporter vectors were assembled in a pGL3-Basic (Promega) backbone (ampicillin resistance gene etc.) using standard cloning techniques. A tetracycline-responsive element (TRE), derived from Clontech's pTRE-d2EGFP vector was inserted into the MCS. In some constructs the luciferase-coding region was replaced with the d1EGFP- or d2EGFP- coding sequence (including Kozak sequence) as defined by Clontech. This was achieved by PCR using appropriate primers with convenient 5' flanking RE sites. In some constructs, specific examples of mRNA destabilising elements were cloned into the 3'UTR-encoding region. Typically, these sequences were prepared by synthesising and then hybridising the sense and antisense sequences. Flanking sequences provided overhanging "sticky ends" that are compatible with those generated when the 3'UTR-encoding region is cut with specific restriction enzymes. Following digestion of the vector with these enzymes and subsequent purification, the hybridised oligomers were ligated into the vector using standard techniques. PCR of genomic DNA or cDNA from an appropriate source was used as an alternative method for obtaining the larger destabilising elements such as c-myc-ARE. Very small elements (e.g., 1 or 2 nonamers) were incorporated into a reverse PCR primer that contained a 5' flanking RE site and a 3' flanking region complementary to the pre-existing 3'UTR in the vector template. Following PCR with an appropriate forward primer (complementary to the protein-coding region and overlapping an endogenous RE site), the PCR product was digested with the

appropriate RE sites and ligated into the original vector.

Nomenclature;

B = Vector backbone derived from Promega's pGL3-Basic

T = Tetracycline-responsive element (TRE), derived from Clontech's pTRE-d2EGFP vector and used as a promoter to drive transcription of the reporter.

G1 = GFP with 1 hr half-life used as reporter i.e., d1EGFP protein encoding sequence as defined by Clontech.

G2 = GFP with 2 hr half-life used as reporter i.e., d2EGFP protein encoding sequence as defined by Clontech.

L = Luciferase used as reporter i.e., The Firefly luciferase encoding sequence from pGL3-Basic (Promega).

R = DsRed2 used as the reporter

R1 = DsRed fused at the carboxy-end to the same MODC mutant as present in d1EGFP

N6 = 6 copies of the nonamer TTATTTATT (SEQ ID NO: 1) inserted into the 3'UTR-encoding region.

N4 = 4 copies of the nonamer TTATTTATT inserted into the 3'UTR-encoding region.

N2 = 2 copies of the nonamer TTATTTATT inserted into the 3'UTR-encoding region.

N1 = 1 copy of the nonamer TTATTTATT inserted into the 3'UTR-encoding region.

fos = The c-fos ARE as defined by Shyu et al (1989) inserted into the 3'UTR-encoding region i.e.,

5'AAAACGTTTTATTGTGTTTTTAATTTATTTATTAAGATGGATTCTCAGATATTTAT ATTTTTATTTTATTTTTTT3'. (SEQ ID NO: 2).

myc = the myc ARE defined as follows

5'ATGCATGATCAAATGCAACCTCACAACCTTGGCTGAGTCTTGAGACTGAAAGAT TTAGCCATAATGTAAACTGCCTCAAATTGGACTTTGGGCATAAAAGAACTTT TTTATGCTTACCATCTTTTTTTTTTCTTTAACAGATTTGTATTTAAGAATTGTTT TTAAAAAATTTTAAGATTTACACAATGTTTCTCTGTAAATATTGCCATTAAAT GTAAATAACTTT3' (SEQ ID NO:21)

Method:

Five micrograms of maxiprep quality DNA was transfected into ~50% confluent 10cm flasks of HeLa Tet-Off cells (Clontech) using Fugene reagent (Roche). ~Ten hours later, the flasks of cells were each split into ~12 small (6cm) dishes and then incubated overnight (~12-14 hrs). At this time point (typically designated time zero or T_0), doxycycline was added to the culture media of most plates at a final concentration of 1 microgram per ml. Cells were trypsinised and collected at this and subsequent time points. For constructs expressing GFP, these samples were analysed by flow cytometry using standard FITC filters. Total GFP fluorescence was measured by gating out non-transfected cells (background fluorescence only) and then multiplying the mean fluorescence per cell (with background fluorescence subtracted) by the number of positive cells. RFP fluorescence (DsRed) was measured similarly using appropriate filters. Cells transfected with luciferase-encoding vectors were lysed and measured in a luminometer using Promega's Dual Luciferase Assay methods and reagents.

Data are typically expressed as the percentage of reporter (fluorescence or luminescence) remaining, relative to time zero.

Since the doxycycline added at time zero causes a block in transcription of the reporter, the rate of decrease in reporter levels indicates the time lag between altered transcription and altered reporter/protein levels. A prime purpose of the invention is to reduce this time lag and Figs 7, 8, 9 and 11-14 demonstrate that this is achieved.

As an example of the utility of this invention, a pharmaceutical company may wish to screen for drugs that reduce transcription of a gene involved in disease. The tetracycline/doxycycline-induced block in transcription from the TRE promoter is a model of such a system. Figs 7 and 8 show that with the standard luciferase reporter vectors, even a total block in transcription (with doxycycline) is not detectable as a decrease in luciferase activity within 10 hrs. The destabilised EGFP mutants represent an improvement in that the total block in transcription is detectable as a 50% decrease in EGFP fluorescence within 11 hrs (d2EGFP; BTG2) or 7 hrs (d1EGFP; BTG1). However, when the latter reporter is combined with an mRNA destabilising element such as 4 copies of the nonamer UUAUUUAUU (BTG1N4), a 50% decrease in

reporter levels is detectable within 3 hrs. It follows that an increase in a transcription would also be detected sooner with constructs containing the destabilising elements (Roth, 1995).

Of course the action of doxycycline is not immediate so that part of the time lag is due to the time required for this drug to induce a 100% transcriptional block. Therefore, the "Effective rate of decay" was measured by plotting data points subsequent to and relative to the time point at 4 hrs after addition of doxycycline (Fig. 9). The effective rate of decay therefore excludes the delay in drug action and is a combined effect of protein and mRNA half-lives. Fig. 9 shows the effective rate of decay with constructs containing 1, 2 or 4 nonamers. These data show that 4 nonamers are more efficient than 2, which is more efficient than 1. Furthermore, these data show that by combining a 1 hr half-life protein (d1EGFP) with 4 nonamers, an effective rate of decay of approximately 1 hr 20 mins can be achieved. This is very close to the 1 hr half-life of the protein and demonstrates an extremely short mRNA half-life. Further reduction could be achieved by combining 2 or more different mRNA instability elements (Fig. 13). However, this is unlikely to be required for most applications. Applications that require a more moderate destabilising effect could utilise 1 or 2 nonamers, rather than 4.

With the standard luciferase reporter, luminescence actually increased after the addition of doxycycline. This is most apparent when the data is expressed on a linear scale (Fig. 8) and can be explained, in part, by the delay in the action of doxycycline. However, even from 4 hrs onwards, no decay is evident, demonstrating the inadequacy of this reporter for measuring changes in transcription over time. A further problem of this vector is revealed in Fig. 10. These data relate to changes in reporter levels over time (24-34 hrs post transfection), in the absence of any treatment or drug. Reporter levels generally increase during the first 24 hrs post transfection as the plasmids enter the cells and begin to be expressed. A decrease is generally seen from about 48 hrs as the plasmids are expelled from the cells. Therefore, measurements are typically taken between 24 and 48 hrs. In the absence of drugs or treatment, the new vector (BTG1N4), containing the instability elements, shows excellent stability of reporter levels. In contrast, the luciferase vector is clearly still ramping up expression levels. Constructs with moderate stability (e.g., BTG1) showed intermediate results. Clearly reporters with longer mRNA and protein half-lives will undergo a more lengthy ramping up phase as indicated in

Fig. 10. The more stable expression levels seen with the new construct during the critical period of 24-34 hrs will facilitate accurate measurement and represent another advantage of the invention.

The rate of decrease in reporter levels can be compared between two or more constructs, which differ in their reporter mRNA sequence (e.g., in 3'UTR) but encode the same protein or different proteins with the same half-life (e.g., d2EGFP, d2EYFP). In this context, differences in the rate of decay indicate an effect of the altered mRNA sequence on mRNA stability. For example, the presence of 4 UUAUUUAUU nonamers as DNA TTATTTATT (SEQ ID NO: 1) (Figs 7-9) or the c-fos ARE (Fig. 11) (SEQ ID NO: 2), within the 3'UTR significantly increased the rate of mRNA decay. In addition to demonstrating the effectiveness of these elements, the methods and vectors used also represent a substantially improved system for detecting other cis-acting mRNA stability/instability elements and this process is also encompassed herein.

As shown in Figures 12 to 14 mRNA destabilising elements work with Luciferase, GFP and DsRed not withstanding the low level of homology between these reporters. DsRed has only 23% homology with EGFP. As shown in Figure 14 *myc* ARE (SEQ ID NO: 21) are effective and are also effective in combination with different destabilising elements.

EXAMPLE 15
mRNA Destabilising Elements

RNA destabilising elements in accordance with the present invention can be derived *inter alia* from the 3'UTR of the following genes. In most cases, the full-length 3'UTR can be used. However, the U-rich and/or AU-rich elements can often be used alone.

a) <u>Phosphoenolpyruvate carboxykinase (PEPCK)</u> mRNA destabilising elements described by Laterza OF *et al.* Regions within 3' half of 3'UTR referred to as JW6 and JW7 i.e., GTATGTTTAAATTATTTTTATACACTGCC CTTTCTTACCTTTCTTTACATAATTGAAATAGGTATCCTGACCA (SEQ ID

NO: 4).

b) The Bicoid gene from *Drosophila melanogaster* comprises an mRNA destabilising element in first 43 nt of 3'UTR (Surdej P. *et al*) such an element can be used *inter alia* to destabilise mRNA in insect cells.

c) The Human Thioredoxin reductase gene (Gasdaska, JR et al). The entire 3'UTR. Nucleotide 1933-3690 (contains 6 AU-rich elements). Segment containing 3 upstream AU repeats (nucleotide 1975-3360). There is also as Non-AU-rich destabilising element at nt 1933-2014.

d) Heat Stable Antigen (HSA) Gene described in Zhou, Q *et al.* For example, nucleotides 1465-1625 in the 3'UTR.

e) Granulocyte-macrophage colony stimulating factor (GM-CSF) ARE described by Chyi-Ying, A *et al.* AGUAAUAUUUAUAUAUUUAUAUUUUUAA AAUAUUU AUUUAUUUAUUUAUUUAA ie as DNA: AGTAAUATTTATATA TTTATATTTTTAAAATATTTATTTATTTATTTATTTAA (SEQ ID NO: 5).

f) c-fos full length 3'UTR or part thereof or ARE as defined by Shyu et al 5'AA AACGTTTTATTGTGTTTTTAATTTATTTATTAAGATGGATTCTCAGATATT TATATTTTTATTTTATTTTTTT3' (SEQ ID NO: 2).

Or by Peng, S *et al.* 5'TTTTATTGTGTTTTTAATTTATTTATTAAGATGGAT TCTCAGATATTTATATTTTTATTTTATTTTTTTT3' (SEQ ID NO: 6).

g) c-jun ARE as described by Peng, S *et al.* 5'UUUCGUUAACUGUGUAUGUA CAUAUAUAUAUUUUUUAAUUUGAUUAAAGCUGAUUACUGUGAAUAAA CAGCUUCAUGCCUUUGUAAGUU3' Sequence as DNA: 5'TTTCGTTAACT GTGTATGTACATATATATATTTTTTAATTTGATTAAAGCTGATTACTGTG AATAAACAGCTTCATGCCTTTGTAAGTT3' (SEQ ID NO: 7).

or the mutant thereof which does not contain a polyadenylation (AAUAAA) signal i.e., 5'UUUCGUUAACUGUGUAUGUACAUAUAUAUAUUUUUUAAUU UGAUUAAAGCUGAUUACUGUGgAUccACAGCUUCAUGCCUUUGUAAGU U3' or as DNA 5'TTTCGTTAACTGTGTATGTACATATATATATTTTTTAA TTTGATTAAAGCTGATTACTGTGgATccACAGCTTCATGCCTTTGTAAGTT 3' (SEQ ID NO: 8).

h) Sequences from the following genes, that include their respective ARE components as described by Henics, T. *et al.*;

<u>IFN-γ ARE</u>
5'UCUAUUUAUUAAUAUUUAACAUUAUUUAUAUAUGGG3' or as DNA 5'TCTATTTATTAATATTTAACATTATTTATATATGGG3' (SEQ ID NO: 9).

i) <u>IL-2 ARE</u>
5'CUCUAUUUAUUUAAAUAUUUAACUUUAAUUUAUUUUUGGAUGUAU UGUUUACUAACUUUUAGUGCUUCCCACUUAAAACAUAUCAGGCUUCU AUUUAUUUAAAUAUUUAAAUUUUAUAUUUAUU3' or as DNA 5'CTCTATTT ATTTAAATATTTAACTTTAATTTATTTTTGGATGTATTGTTTACTAACTTT TAGTGCTTCCCACTTAAAACATATCAGGCTTCTATTTATTTAAATATTTA AATTTTATATTTATT3' (SEQ ID NO: 10).

j) <u>c-myc ARE (see also SEQ ID NO:21)</u>
5'AUAAACCCUAAUUUUUUUAUUUAAGUACAUUUUGCUUUUAAAGUU3' or as DNA 5'ATAAACCCTAATTTTTTTATTTAAGTACATTTTGCTTTTAAAG TT3' (SEQ ID NO: 11).

k) <u>IL-10</u>
5'UAGAAUAUUUAUUACCUCUGAUACCUCAACCCCCAUUUCUAUUUAU

UUACUGAGCUUCUCUGUGAACGAUUUAGAAAGAAGCCCAAUAUUAUA AUUUUUUUCAAUAUUUAUUAUUUUCA3' or as DNA 5'TAGAATATTTATT ACCTCTGATACCTCAACCCCCATTTCTATTTATTTACTGAGCTTCTCTGTG AACGATTTAGAAAGAAGCCCAATATTATAATTTTTTTCAATATTTATTAT TTTCA3' (SEQ ID NO: 12).

l) bcl-2

Sequences from the bcl-2 3'UTR that include all or part of the bcl-2 ARE as defined by Schiavone, N *et al.* 5'UCAGCUAUUUACUGCCAAAGGGAAAUA UCAUUUAUUUUUUACAUUAUUAAGAAAAAAGAUUUAUUUAUUUAAGA CAGUCCCAUCAAAACUCCGUCUUUGGAAAUC3' (M13994 from nt 2371-2475) or as DNA 5'TCAGCTATTTACTGCCAAAGGGAAATATCATTTATTT TTTACATTATTAAGAAAAAAGATTTATTTATTTAAGACAGTCCCATCAAA ACTCCGTCTTTGGAAATC3' (SEQ ID NO: 13).

m) TNF ARE as described by Xu, N *et al.* 5'AUUAUUUAUUAUUUAUUUAUUA UUUAUUUAUUUA3' or as DNA 5'ATTATTTATTATTTATTTATTATTTATTT ATTTA3' (SEQ ID NO: 14).

n) IL3 ARE as described by Xu, N *et al.* 5'UAUUUUAUUCCAUUAAGGCUAUU UAUUUAUGUAUUUAUGUAUUUAUUUAUUUAUU3' or as DNA 5'TATTTT ATTCCATTAAGGCTATTTATTTATGTATTTATGTATTTATTTATTTATT3' (SEQ ID NO: 15).

o) The nonamer UUAUUUAUU as DNA TTATTTATT (SEQ ID NO: 1) As described by Zubiaga, A *et al.*

p) The nonamer UUAUUUA(U/A)(U/A) as DNA TTATTTA(T/A)(T/A) (SEQ ID NO: 3) as described by Lagnado, C *et al.*

q) The pentamer AUUUA as described by Xu, N *et al.* or as DNA ATTTA (SEQ ID

NO: 16)

r) The tetramer AUUU or as DNA ATTT (SEQ ID NO: 17).

AU-rich elements (AREs) in general of both class I and class II as described by Chen, C and Shyu, A.

Plants have DST (downstream sequences) which act as destabilising elements. DST sequence are defined in: Newnan, T *et al.* A proposed consensus DST sequence is:

G**G**AgN$_{2\text{-}9}$cA**TAGAT**TaN$_{3\text{-}8}$(A/C)(T/A)(A/T)Ttt**GTA**(T/C)

s) This is based on comparison of 9 different DST sequences.
Bold = conserved in 9/9 genes.
Capital = conserved in at least 7/9 genes
N2-9 = variable length region of 2-9 nucleotides; average = 5.
N3-8 = variable length region of 3-8 nucleotides; average = 6.
Distance from stop codon = 19-83 nt.

Further examples of DST sequences include the:

Soybean 10A5 gene;
5'GGAGN$_5$CATAGATTAN$_8$AAATTTGTAC3' (SEQ ID NO: 18).

Arabidopsis SAURAC1 gene;
5'GGAAN9CATAGATCGN$_8$CAATGCGTAT3' (SEQ ID NO: 19).

DST sequences are an alternative to AU-rich elements for use in plants. Both AU-rich elements and DST sequences destabilise transcripts in plants.

t) Iron Responsive Element (IRE)
Thomson, A *et al.* 1999.

IREs contain consensus CAGUG in a hairpin-loop.

Examples:

Ferritin IRE;

GUUCUUGCUUCAA**CAGUG**UUUGAACGGAAC or as DNA GTTCTTGCTTCA A**CAGTG**TTTGAACGGAAC (SEQ ID NO: 20).

Transferrin Receptor IRE;

GAUUAUCGGGAG**CAGUG**UCUUCCAUAAUC or as DNA GATTATCGGGAG **CAGTG**TCTTCCATAATC (SEQ ID NO: 21).

Iron Regulatory Proteins (IRPs; e.g., IRP1 and 2) bind IREs in an iron-dependent fashion. Binding is also modulated by various other stimuli and treatments (e.g., oxidative stress, nitric oxide, erythropoietin, thyroid hormone or phosphorylation by PKCs.

IREs can modulate both translational efficiency and mRNA stability. For example, the 5'UTR IRE in Ferritin mRNA blocks translation only when bound to an IRP. The IREs in the 3'UTR of Transferrin receptor mRNA inhibit mRNA decay when bound by an IRP. Therefore, IREs can be inserted into 5'UTR or 3'UTR of expression vectors to provide expression that can be controlled by modulating iron levels or other stimuli.

Destabilising elements can be used with Clontech's Mercury Pathway Profiling vectors and *in vivo* kinase assay kits. Clontech produce 3 different protein destabilising elements, all containing a PEST sequence and all derived from the MODC gene. Different mutant MODCs placed at the carboxy-end of EGFP provide protein half-lives of 1 hr, 2 hr and 4 hr. mRNA destabilising elements in accordance with the present invention can be used in conjunction with these and any other protein destabilising element (e.g., ubiquitination signals).

u) c-myc ARE may also be defined as: 5'ATGCATGATCAAATGCAACCTCAC AACCTTGGCTGAGTCTTGAGACTGAAAGATTTAGCCATAATGTAAACTG

CCTCAAATTGGACTTTGGGCATAAAAGAACTTTTTATGCTTACCATCTT TTTTTTTTCTTTAACAGATTTGTATTTAAGAATTGTTTTTAAAAAATTTTA AGATTTACACAATGTTTCTCTGTAAATATTGCCATTAAATGTAAATAACT TT3' (SEQ ID NO: 22).

Another useful mRNA element can be obtained from histone mRNA, Specifically, 3'UTR sequences including a consensus stem loop structure are described by Gallie, D et al: TGA-$N_{20\text{-}40}$-CCAAAGGYYYUUYUNARRRCCACCCA, where Y=pyrimidine, R=purine, N= any nucleotide or as DNA TGA-$N_{20\text{-}40}$-CCAAAGGYYYTTYTN ARRRCCACCCA (SEQ ID NO: 23).

Such sequences can increase translational efficiency. Moreover, they are capable of directing mRNA decay specifically outside of S phase. Reporter constructs containing a cell-cycle-specific promoter, together with mRNA destabilising elements are contemplated in this invention as a tool for directing cell-cycle specific expression (e.g., of a reporter). The histone 3'UTR element offers an alternative for use with an S-phase or late G1 specific promoter, since it will direct increased mRNA decay in G2 relative to S-phase, thus further restricting protein expression to S phase.

Yet another use of 3'UTR elements in expression vectors is for the purpose of specifically localising the chimeric mRNA. For example, the utrophin 3'UTR is capable of directing reporter mRNA to the cytoskeletal-bound polysomes. mRNA stabilising elements are also contained in this 3'UTR (Gramolini, A, et al)

EXAMPLE 16

mRNA Stabilising Elements and Expression Vectors encoding a stabilised mRNA

Stabilising sequences may contain CT-rich elements and/or sequences derived from long-lived mRNAs (particularly 3'UTR regions)

CT-rich elements may contain (C/U)CCAN$_x$CCC(U/A)Py$_x$UC(C/U)CC as described by Holcik and Liebhaber, 1997.

CT-rich elements may contain the following element CCTCCTGCCCGCTGGGCCTCCCAACGGGCCCTCCTCCCCTCCTTGC or parts thereof, including CCTCC, CCTCCTGCC or CCCTCCTCCCCTGG

A 14 nt pyrimidine-rich region from the 3'UTR of human beta-globin described by Yu and Russell is also contemplated for use as a stabilising element.

Examples of long-lived mRNAs from which stabilising elements may be derived include; Alpha2 globin, Alpha1 globin, beta globin. From human, mouse, rabbit or other species, bovine growth hormone 3'UTR.

The mRNA instability elements described herein generally act in a dominant-fashion to destabilise chimeric genes. It follows, therefore that mRNA stabilising elements are often recessive-acting. For example, insertion of a c-fos ARE into the rabbit beta-globin gene, results in a destabilised transcript despite the continued presence of mRNA stability elements (Shyu, A *et al.* 1989). Both alpha- and beta-globin mRNAs contain stability elements that have been mapped to their respective 3'UTRs, whereas zeta-globin mRNA lacks these elements and is less stable. Replacing the zeta-globin 3'UTR with that of alpha globin mRNA nearly doubles mRNA stability (Russell, J *et al.* 1998). However, such elements do not stabilise all transcripts. Therefore, the requirements for generating an expression vector that expresses a stable mRNA differ, dependent on the original mRNA that is to be stabilised. To create such a vector it is generally preferable to include large segments from a stable gene such as alpha- or beta-globin. With these examples, such segments should preferably include the entire globin 3'UTR, replacing the endogenous 3'UTR. As exemplified with zeta-globin, this is sometimes sufficient. However, the further incorporation of protein-coding and/or 5'UTR sequences is often required. Generally, it is preferable to replace any endogenous AU- or U-rich regions, which may act as dominant destabilising elements (these can be identified using the techniques described herein). Such regions in the 5'UTR or 3'UTR are simply replaced with alpha- or

beta-globin sequences from the same relative position. Instability elements from the coding region can be rendered non-functional by mutation to synonymous codons. The globin protein-coding region can be incorporated into the coding region of the gene of interest to create an N- or C-terminal fusion protein. However this is often not desirable and it is generally sufficient to localise the globin protein-coding region (and 3'UTR) into the 3'UTR of the chimeric gene. This allows expression of the desired protein from a more stable transcript, thus markedly increasing levels of the protein. When the desired protein is a reporter or is fused to a reporter or can be easily distinguished from endogenous protein, the TRE vector system described herein (see Fig. 7) greatly facilitates the testing of chimeric constructs for mRNA stability.

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

TABLE 1

Signal transducers that could be used in the present invention

Signal transducer
AKT (also called PKB)
Fas L / BID
JAK 7 Stat
MKK-47 / JNK
MTOR / p70 s6 kinase
NFκB
p38
PKA / Rap1 B-raf
Ras / Raf
Wnt / GSK3
Erk 1&2

BIBLIOGRAPHY

Chen, C and Shyu, A. Trends Biochem. Sci. 1995: vol 20 465-470.

Chyi-Ying, A *et al.* MCB 1995: vol 15 (10) 5777-5788.

Contag, *et al.* Non-invasive localization of a light-emitting conjugate in a mammal United States Patent 5,650,135. July 22, 1997.

Cormack, *et al.* FACS-optimized mutants of the green fluorescent protein (GFP) United States Patent 5,804,387. September 8, 1998.

Dandekar T *et al* Bioinformation 1998: *14*(3):271-278.

Darzynkiewicz. Laser-scanning cytometry: A new instrumentation with many applications. Exp Cell Res 1999;249:1-12.

Gallie, D *et al.* Nucleic Acids Res. 1996: vol24 (10); 1954-1962.

Gasdaska, JR *et al.* JBC 1999:vol 274 (36); 25379-25385.

Gossen, *et al.* Tight control of gene expression in eucaryotic cells by tetracycline-responsive promoters. United States Patent 5,464,758. November 7, 1995.

Gramolini, A, *et al.* JBC 2001: vol 154 (6) 1173-1183.

Henics, T. et al JBC 1999: vol 274 (24) 17318-17324.

Holcik and Liebhaber, 1997, PNAS vol 94 pp2410-2414.

Huet, X *et al.* Mol Cell Biol. 1996; vol 16 (7): 3789-3798.

Ibrahim, *et al.* Pitfall of an internal control plasmid: Response of *Renilla* luciferase (pRL-TK) plasmid to dihydrotestosterone and dexamethasone. Biotechniques 2000;29:682-684.

Kamentsky *et al.* Slide-based laser scanning cytometry. Acta Cytol 1997;41:123-43.

Li, *et al.* Generation of destabilised green fluorescent protein as a transcription reporter. J Biol Chem 1998;273:34970-34975.

Lagnado C *et al*, MCB, *14*:7984-7995, 1994.

Laterza, OF *et al* Am J Physiol Renal Physiol. 2000: vol 279(5) F866-F873.

Leclerc G et al, Biotechniques 2000, *29*(3) 590-595.

Lee, H *et al.* Gene Expr 1995; vol 4(3): 95-109.

Li, *et al.* Rapidly degrading GFP-fusion proteins. United States Patent 6,130,313 October 10, 2000.

Liu, J *et al.* JBC 2000; vol 275 (16): 11846-11851.

Newnan, T *et al.* The Plant Cell 1993: vol 5; 701-714.

Peng, S *et al.* MCB 1996: vol 6 (4) 1490-1499.

Ross, J. Microbiological Reviews. 1995: vol 59 (3):423-450.

Saito, T *et al.* Biochem Biophys Res Commun. 1998; vol 252 (3): 775-778.

Schiavone, N *et al.* FASEB J. 2000: vol 14 174-184.

Shyu et al 1989:Genes & Dev. Vol 3, pp60-72.

Stein, J *et al.* Int J Obes Relat Metab Disord. 1996 Mar;20 Suppl 3: S84-90.

Surdej, P and Jacobs-Lorena, M. MCB 1998 vol 18 (5) 2892-2900.

Thomson, A *et al.* Int. J Biochem Cell Biol. 1999: vol 31; 1139-1152.

Tsien, *et al.* Modified green fluorescent proteins. United States Patent 5,625,048. April 29, 1997.

Tsien, *et al.* Modified green fluorescent proteins United States Patent 5,777,079. July 7, 1998.

Vazhappilly, R and Sucher, N. Neurosci Lett. 2002; vol 318 (3): 153-157.

Xu W *et al*, MCB, *17*(8):4611-4621, 1997.

Yu and Russell MCB Sept 2001 5879-88.

Zhou, Q *et al.* MCB 1998: vol 18 (2) 815-826.

Zubiaga *et al.* MCB 1995;15:2219-30.

CLAIMS

1. An expression vector comprising a transcribable polynucleotide which comprises a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide.

2. An expression vector according to claim 1 wherein said RNA element is a destabilising element which reduces the stability of said transcript.

3. An expression vector according to claim 1 wherein said RNA element is a stabilising element which increases the stability of said transcript.

4. An expression vector according to claim 2 wherein said transcribable polynucleotide comprises a polynucleotide of interest and encodes a polypeptide.

5. An expression vector according to claim 4 wherein said polypeptide comprises a reporter protein.

6. An expression vector according to claim 2 wherein said transcribable polynucleotide comprises a sequence of nucleotides encoding a reporter protein.

7. A vector according to claim 1 or 2 comprising one or more members selected from the group consisting of:

(i) a multiple cloning site for introducing a sequence of nucleotides;

(ii) a reporter gene;

(iii) a promoter and/or enhancer for regulating expression of said transcribable polynucleotide;

(iv) a polyadenylation sequence;

(v) a selectable marker gene; and

(vi) an origin of replication.

8. A vector according to claim 7 further comprising at least one site which is cleavable enzymatically or otherwise biochemically to provide a linearised vector into which PCR amplification products are clonable directly.

9 A vector according to claim 2 wherein the sequence of nucleotides encoding said destabilising element is, or is derived from, a gene selected from *c-fos, c-jun, c-myc, GM-CSF, IL-3, TNF-alpha, IL-2, IL-6, IL-8, IL-10, Urokinase, bcl-2, SGLT1 (Na(+)-coupled glucose transporter), Cox-2 (cyclooxygenase 2), IL-8, PAI-2 (plasminogen activator inhibitor type 2), beta1-adrenergic receptor* or *GAP43.*

10. A vector according to claim 3 wherein the sequence of nucleotides encoding said stabilising element is, or is derived from, a gene selected from *alpha2 globin, alpha1 globin, beta globin, or growth hormone, erythropoietin, ribonucleotide reductase R1* or *m1 muscarinic acetylcholine.*

11. A vector according to claim 5 wherein the sequence of nucleotides encoding said destabilising element is selected from any one of SEQ ID NOS 1 to 23, or biologically active fragments thereof, or variants or derivatives of these.

12. A vector according to claim 11 wherein the sequence of nucleotides encoding said destabilising element is selected from SEQ ID NO: 1, 2 or 22 , or biologically active fragments thereof, or variants or derivatives of these.

13. A vector according to claim 5 or 6 wherein said reporter protein is selected from Luciferase, Green Fluorescent Protein, Red Fluorescent Protein, SEAP, CAT, or biologically active fragments thereof, or variants or derivatives of these.

14. A vector according to claim 4 wherein said polypeptide comprises a protein destabilising element.

15. A vector according to claim 13 wherein said reporter protein comprises a protein destabilising element.

16. A vector according to claim14 or 15 wherein said protein destabilising element is encoded by a sequence of nucleotides encoding a PEST sequence or a ubiquitin or a biologically active fragment thereof, or variant or derivative of these.

17. A cell comprising one or more vectors according any one of the preceding claims.

18. A cell according to claim 17 wherein said cell is a eukaryotic cell.

19. A cell according to claim 18 wherein said cell is a mammalian cell.

20. A cell according to claim 19 wherein said cell is a human cell.

21. A genetically modified non-human organism comprising one or more of the vectors according to any one of claims 1 to 16.

22. A method for determining expression of a polynucleotide of interest, said method comprising expressing said polynucleotide of interest from an expression vector for a time and under conditions sufficient for RNA and protein synthesis to occur, said vector comprising a transcribable polynucleotide which comprises said polynucleotide of interest and a sequence of nucleotides encoding an RNA element which modulates the stability of a transcript corresponding to said transcribable polynucleotide; and wherein said expression vector

comprises one or more members selected from the group consisting of:

(i) a multiple cloning site for introducing a sequence of nucleotides;

(ii) a reporter gene;

(iii) a promoter and/or enhancer for regulating expression of said transcribable polynucleotide ;

(iv) a polyadenylation sequence;

(v) a selectable marker gene; and

(vi) an origin of replication;

and measuring the level and/or functional activity of an expression product of the transcribable polynucleotide over time compared to a control wherein said element enhances the temporal correlation between the activity of the promoter and/or enhancer that is operably connected to said transcribable polynucleotide and the level and/or functional activity of said expression product.

23. A method according to claim 22 wherein the transcribable polynucleotide comprising a sequence of nucleotides encoding said RNA destabilising element is, or is derived from, a gene selected from *c-fos, c-jun, c-myc, GM-CSF, IL-3, TNF-alpha, IL-2, IL-6, IL-8, IL-10, Urokinase, bcl-2, SGLT1 (Na(+)-coupled glucose transporter), Cox-2 (cyclooxygenase 2), IL-8, PAI-2 (plasminogen activator inhibitor type 2), beta1-adrenergic receptor* or *GAP43.*

24. A method according to claim 22 wherein the transcribable polynucleotide encoding said RNA destabilising element comprises a sequence of nucleotides as set forth in any one of SEQ ID NOS 1 to 23, or a biologically active fragment thereof, or variants or derivatives of these.

25. A method according to claim 22 wherein the transcribable polynucleotide encoding said RNA destabilising element comprises a sequence set forth in SEQ ID NO: 1, 2 or 22, or a biologically active fragment thereof, or variants or derivatives of these.

26. A method for identifying a nucleotide sequence encoding an RNA element which modulates the stability of an RNA transcript, said method comprising introducing a test nucleotide sequence into an expression vector whereby said nucleotide sequence is connected to a polynucleotide encoding a reporter protein to form a transcribable polynucleotide which is operably connected to a promoter and/or enhancer; expressing said transcribable polynucleotide for a time and under conditions sufficient for RNA and protein synthesis to occur; and wherein said expression vector comprises one or more members selected from the group consisting of:

(i) a multiple cloning site for introducing said test nucleotide sequence;

(ii) a polyadenylation sequence;

(iii) a selectable marker gene; and

(iv) an origin of replication;

and measuring the level and/or functional activity of an expression product of said transcribable polynucleotide over time compared to that of a control vector in the absence of said nucleotide sequence, wherein a level and/or functional activity which is different to that of the control vector over that time is indicative of a nucleotide sequence that encodes said RNA element.

27. The method of claim 26, wherein the promoter is modulatable.

28. The method of claim 27, wherein the promoter is inducible.

29. The method of claim 27, wherein the promoter is repressible.

30. The method of claim 26, wherein the expression product whose level and or functional activity is measured is said reporter protein.

31. The method of claim 26, wherein said reporter protein is selected from Luciferase, Green Fluorescent Protein, Red Fluorescent Protein, SEAP, CAT, or biologically active fragments thereof, or variants or derivatives of these.

32. The method of claim 31, wherein said reporter protein comprises a protein destabilising element.

33. The method of claim 32, wherein said protein destabilising element is encoded by a sequence of nucleotides encoding a PEST sequence or a ubiquitin or a biologically active fragment thereof, or variant or derivative of these.

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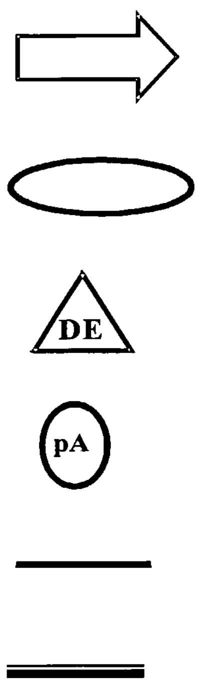
Figure 1.

Expression Vectors Encoding a Destabilised mRNA



Any promoter or promoter element

Protein or polypeptide encoding region

mRNA destabilising element

Polyadenylation signal

5'-untranslated region (5'-UTR)

3'-untranslated region (3'-UTR)

Figure 2.

Transcription Reporter Vectors

Fig. 2a. Vector Series 2

Fig. 2b. Vector Series 3

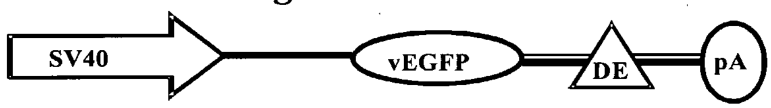
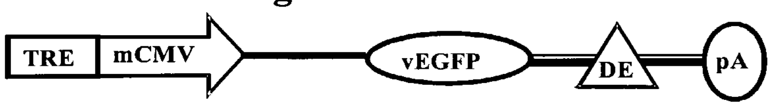
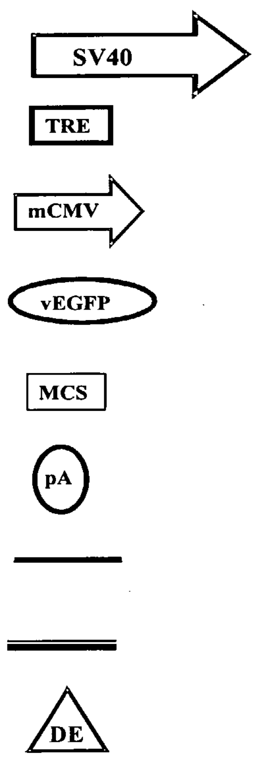


Fig. 2c. Vector Series 4





SV40 or other constitutive promoter

Tetracycline Responsive Element

Minimal Cytomegalovirus (CMV) promoter

Region encoding any variant of enhanced green fluorescent protein (EGFP). Each vector in the series encodes a different variant.

Multiple Cloning Site (any site useful for inserting DNA fragments)

Polyadenylation signal

5'-untranslated region (5'-UTR)

3'-untranslated region (3'-UTR)

mRNA destabilising element

3/14

Figure 3.

Bi-directional Transcription Reporter Vectors

Fig. 3a. Vector Series 5

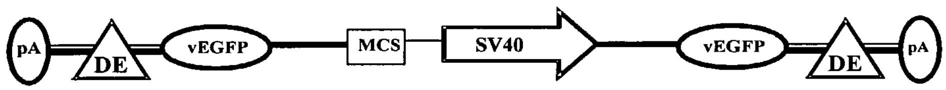
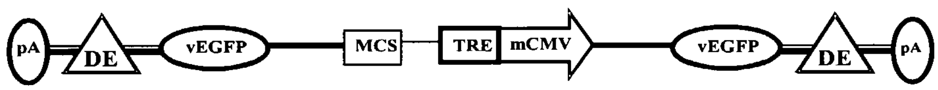
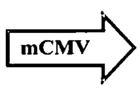


Fig. 3b. Vector Series 6



SV40 or other constitutive

Tetracycline Responsive



Minimal Cytomegalovirus (CMV)

Region encoding anyenhanced green fluorescent proteir
Each vector in the series encodes a

Multiple Cloning Site (any site useful for inserting

Polyadenylation

5'-untranslated region (5'-

3'-untranslated region (3'-

mRNA destabilising

Spacer region to separate promoters. Can contain additional polyadenylation signals.

Figure 4.

Reporter Vectors For Studying Post-transcriptional Regulation

Fig. 4a. Vector Series 7

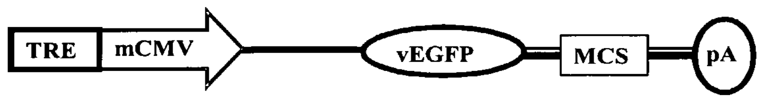
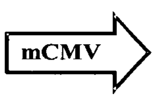


Fig. 4b. Vector Series 8

Tetracycline Responsive Element



Minimal Cytomegalovirus (CMV) promoter

Region encoding any variant ofenhanced green fluorescent protein (EGFP)
Each vector in the series encodes a different variant.

Multiple Cloning Site (any site useful for inserting DNA fragments)

Polyadenylation signal

5'-untranslated region (5'-UTR)

3'-untranslated region (3'-UTR)

Figure 5.

Evidence For Errors Associated with Co-transfection in Luciferase-based

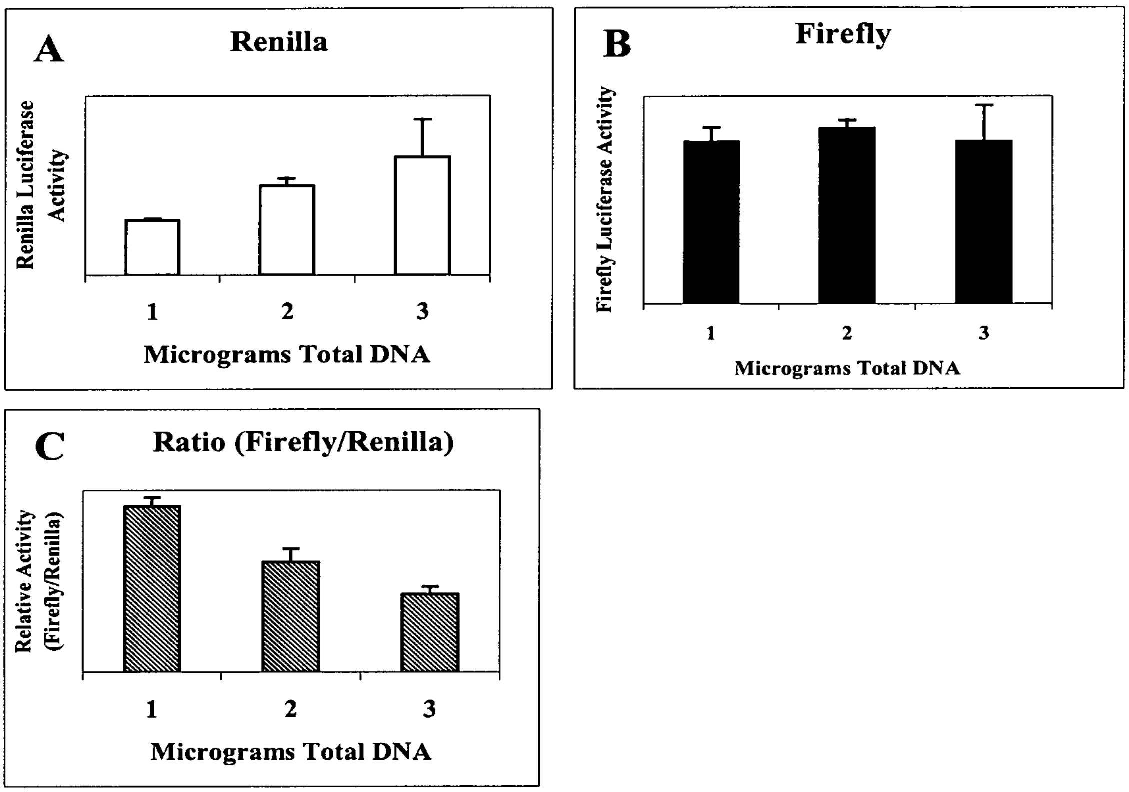


Figure 6.

Evidence For Errors Associated with Dual Luciferase Assay.

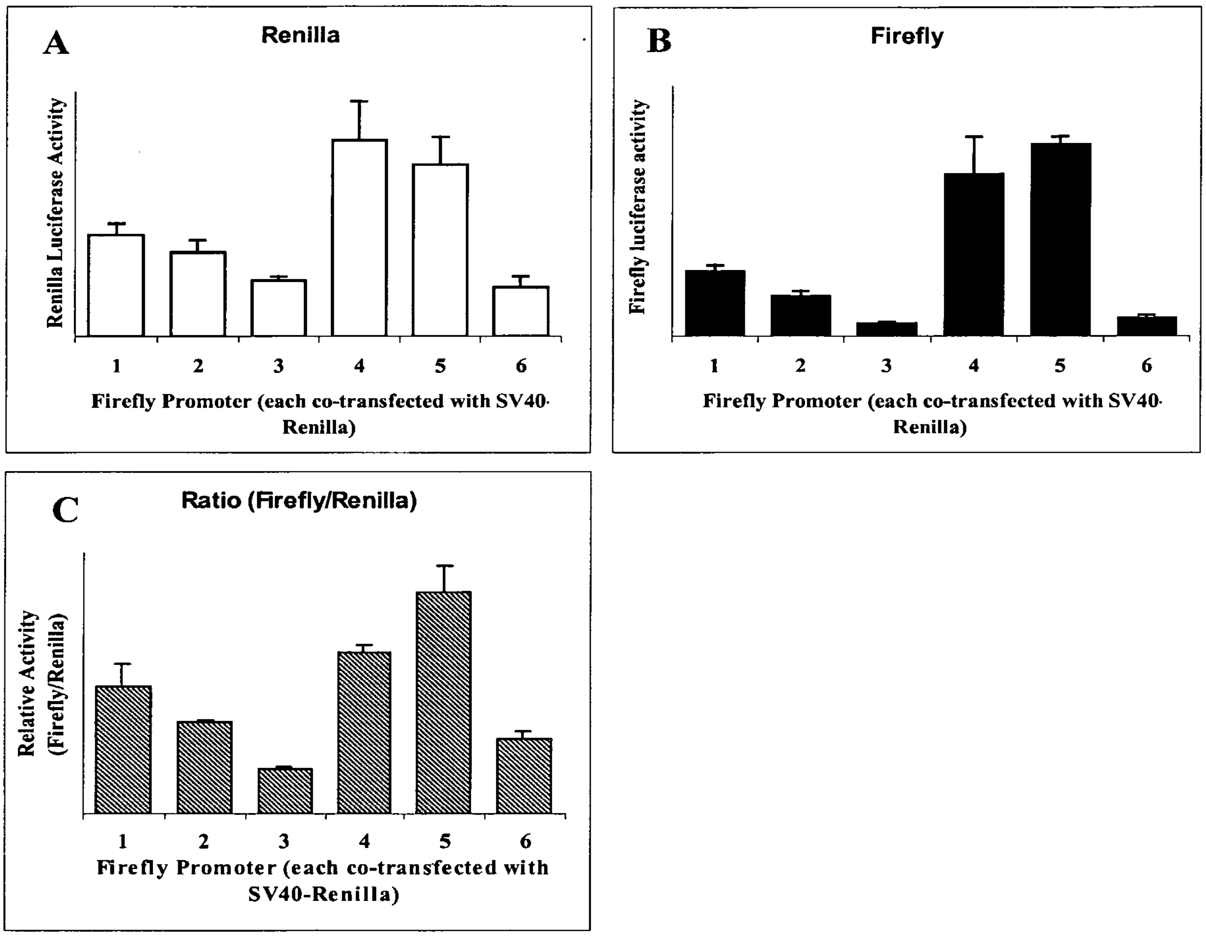


Figure 7.

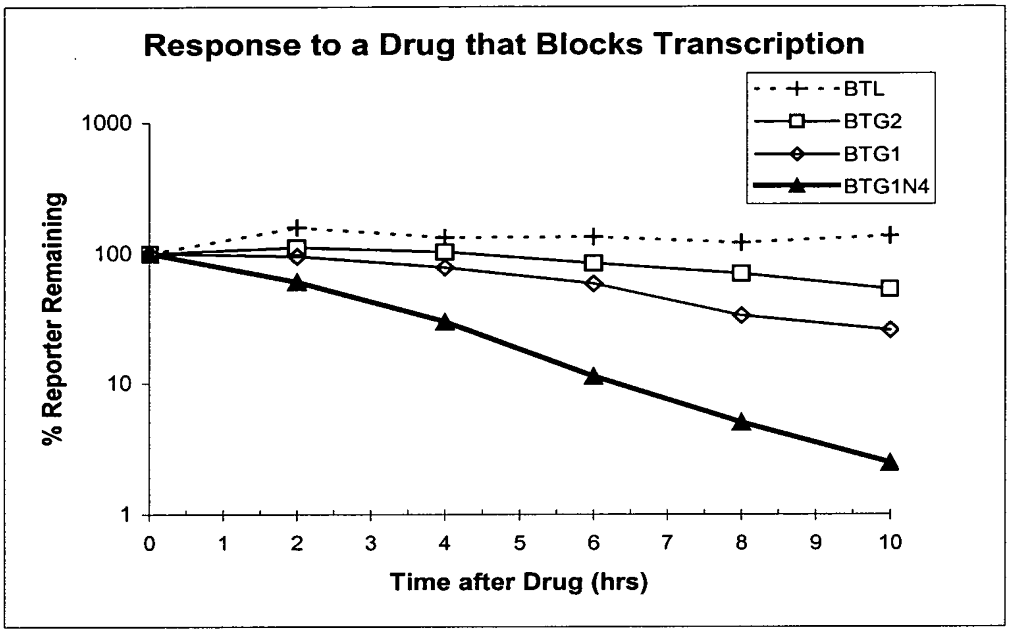

Response to a Drug that Blocks Transcription
BTL
BTG2
BTG1
BTG1N4
1000
100
10
1
% Reporter Remaining
0
1
2
3
4
5
6
7
8
9
10
Time after Drug (hrs)

Figure 8.

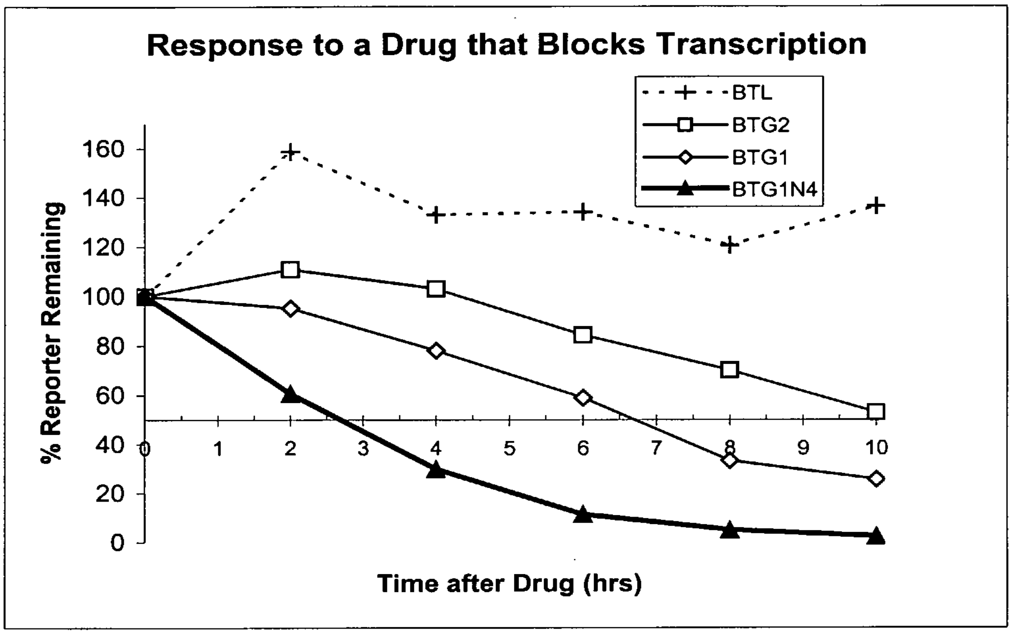

Response to a Drug that Blocks Transcription
BTL
BTG2
BTG1
BTG1N4
% Reporter Remaining
0
20
40
60
80
100
120
140
160
0
1
2
3
4
5
6
7
8
9
10
Time after Drug (hrs)

Figure 9.

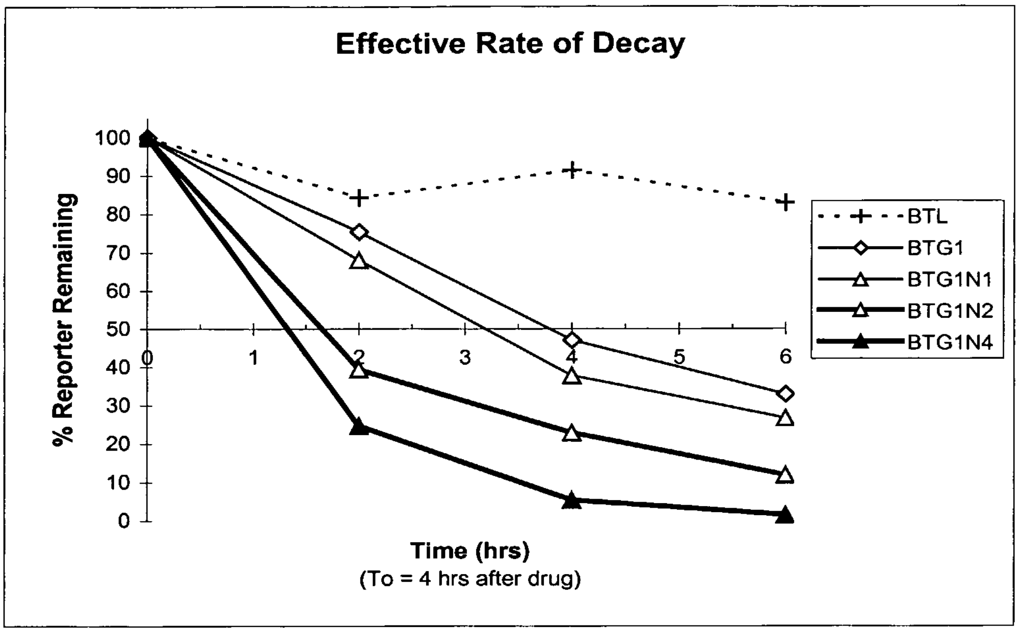

Effective Rate of Decay
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
% Reporter Remaining
Time (hrs)
(To = 4 hrs after drug)
BTL
BTG1
BTG1N1
BTG1N2
BTG1N4

Figure 10

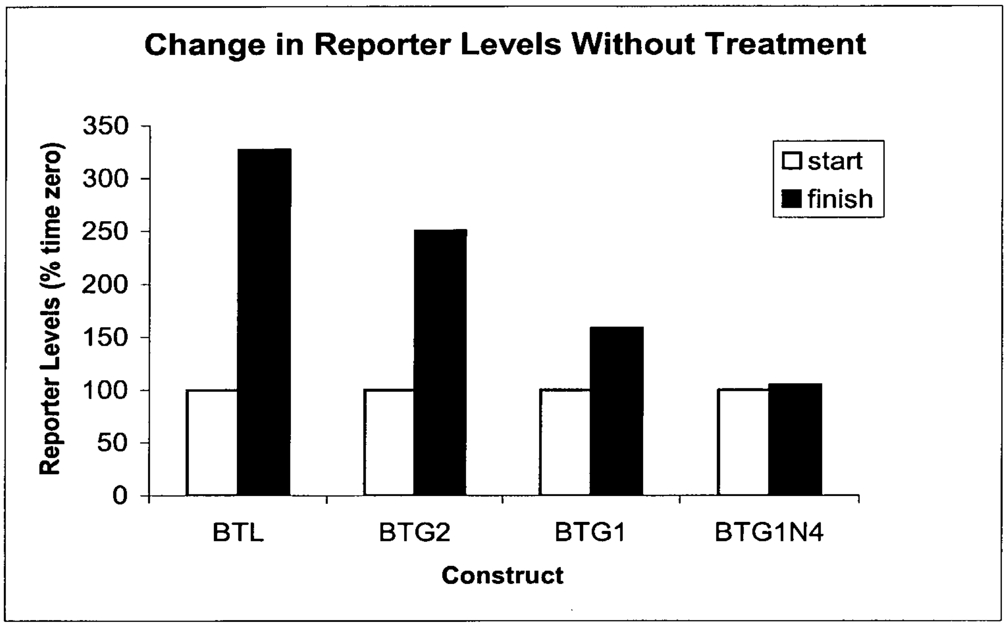

Change in Reporter Levels Without Treatment
Reporter Levels (% time zero)
350
300
250
200
150
100
50
0
start
finish
BTL
BTG2
BTG1
BTG1N4
Construct

Figure 11

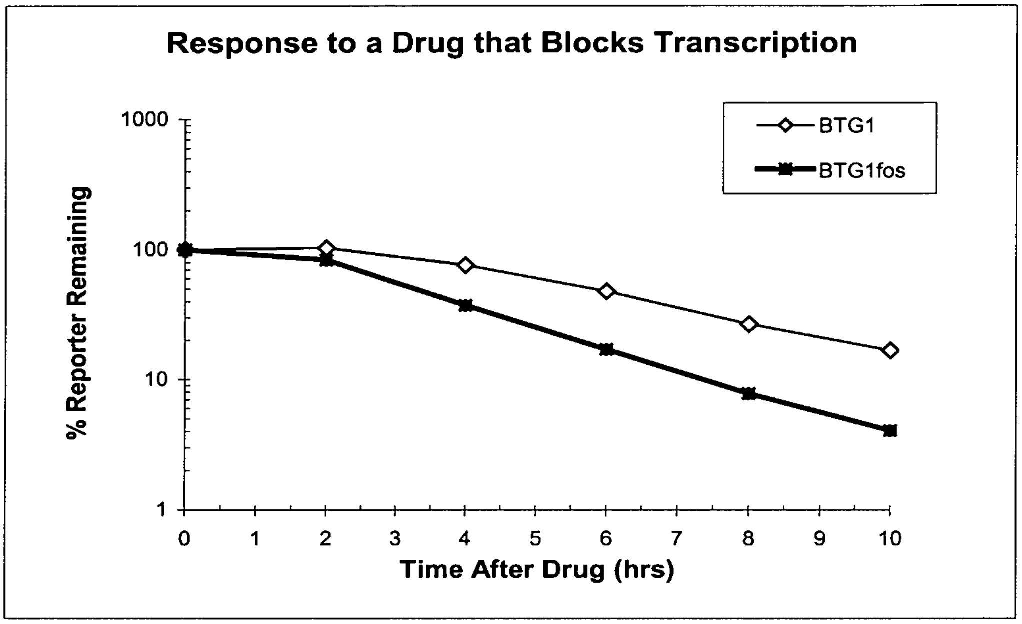
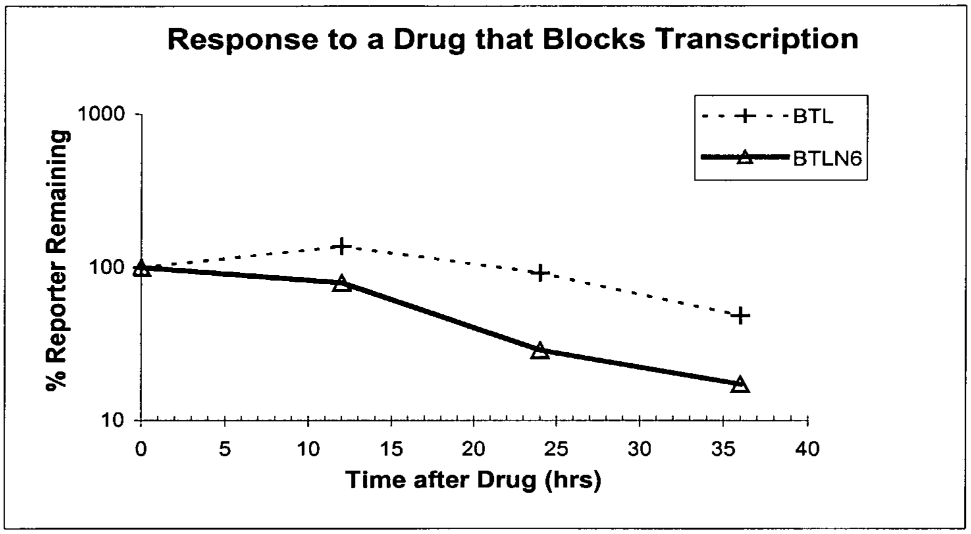

Response to a Drug that Blocks Transcription
BTG1
BTG1fos
% Reporter Remaining
1000
100
10
1
0
1
2
3
4
5
6
7
8
9
10
Time After Drug (hrs)

Figure 13



Response to a Drug that Blocks Transcription
% Reporter Remaining
1000
100
10
0
5
10
15
20
25
30
35
40
Time after Drug (hrs)
BTL
BTLN6

Figure 13

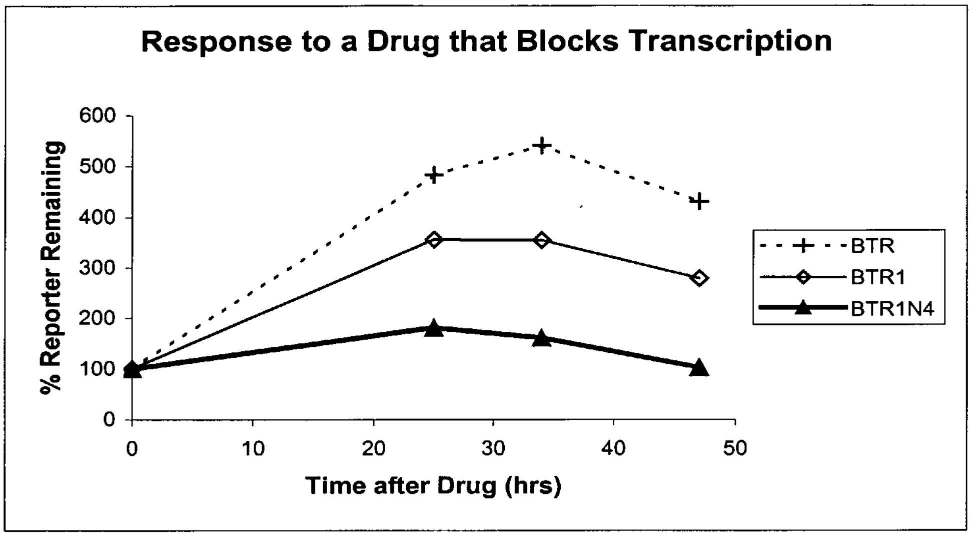
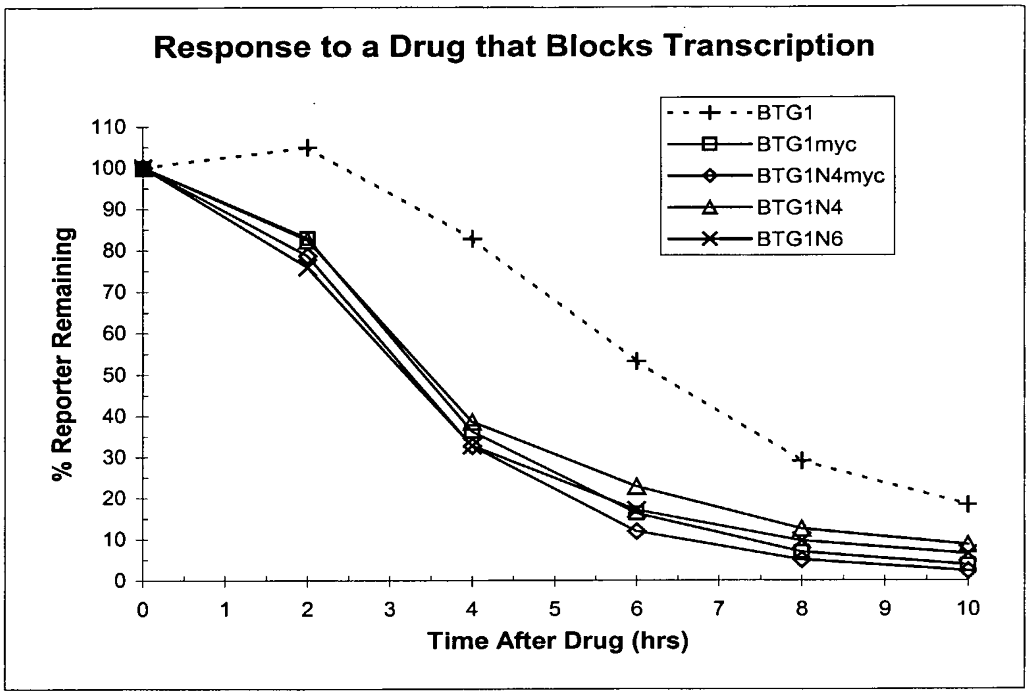

Response to a Drug that Blocks Transcription
% Reporter Remaining
600
500
400
300
200
100
0
0
10
20
30
40
50
Time after Drug (hrs)
BTR
BTR1
BTR1N4

Figure 14



Response to a Drug that Blocks Transcription
BTG1
BTG1myc
BTG1N4myc
BTG1N4
BTG1N6
% Reporter Remaining
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
Time After Drug (hrs)

SEQUENCE LISTING

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<120> Novel Expression Vectors

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ttattttatt ttttt 75

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<400> 3
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ggtatcctga cca 73

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<400> 5
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tatttttttt 70

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gaataaacag cttcatgcct ttgtaagtt 89

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<212> DNA
<213> Mammalian

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ggatccacag cttcatgcct ttgtaagtt 89

<210> 9
<211> 36
<212> DNA
<213> Mammalian

<400> 9
tctatttatt aatatttaac attatttata tatggg 36

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       R is purine
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/00351

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. 7: C12N 15/67

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN (WPIDS): untranslated region and (destab? or stab?)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	WO 99/14346, A (Sequitur, Inc) 25.03.99; see pages 4 and 11-25.	1-10; 22 and 26
X,Y	WO 95/29244, A (Wisconsin Alumni Research Foundation) 02.11.95; see page 5 and claims	1-10, 22 and 26
X,Y	Oncogene 11, pp 2127-2134 (1995) Veyrune et al "c-fos mRNA instability determinants present within both the coding and the 3' non coding region link the degradation of this mRNA to its translation".	1-10, 22 and 26

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search 10 May 2002	Date of mailing of the international search report 17 MAY 2002
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer MADHU K. JOGIA Telephone No : (02) 6283 2512

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/00351

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	Nucleic Acids Research 27, pp 1164-1673 (1999) Maurer et al "An AU-rich sequence in the 3'-UTR of plasminogen activator inhibitor type 2 (PAI-2) mRNA promotes PAI-2 mRNA decay and provides a binding site for nuclear HuR"	1-10, 22 and 26
X,Y	Molecular and Cellular Biology 21, pp 721-730 (Feb 2001) Dean et al "The 3' Untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR".	1-10, 22 and 26
X	J Biol Chem 275, pp 30248-30255 (2000) Provost et al "Length increase of the human α-globin 3'-untranslated region disrupts stability of the pre-mRNA but not that of the mature mRNA"	1-10, 22 and 26
X	J Biol Chem 275, pp 12963-12969 (2000) Short et al "Structural determinants for post-transcriptional stabilization of lactate dehydrogenase A mRNA by the protein kinase C signal pathway"	1-10, 22 and 26
X	J Biol Chem 273, pp 15749-15757 (1998) Yeilding et al "c-myc mRNA is down-regulated during myogenic differentiation by accelerated decay that depends on translation of regulatory coding elements".	1-10, 22 and 26
X	Molecular and Cellular Biology 17, pp 2698-2707 (1997) Yeilding et al "Coding elements in exons 2 and 3 target c-myc mRNA downregulation during myogenic differentiation".	1-10, 22 and 26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU02/00351

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9914346	AU	93193/98	CA	2304982	EP	1021549
WO	9529244	AU	22959/95	US	5587300		

END OF ANNEX